R E M A R K S

Presently Claimed Invention

The present claims are directed to a method for detecting colon cancer wherein the tumor marker is COX-2. The method comprises: a) homogenizing collected feces in the presence of an RNase inhibitor to prepare a suspension thereof, without separating cell components from the feces; b) extracting RNA from the suspension from step a) to provide extracted RNA; c) carrying out reverse transcription on the extracted RNA from step b) to provide cDNA; d) amplifying the cDNA from step c) and e) detecting the amplified COX-2 from step d) (see applicant's claim 5).

Anticipation Rejection Under 35 USC 102

Claims 5, 17 to 18, 20, 22 and 23 were rejected under 35 USC 102 as being anticipated by Chapkin et al. (USP 6,258,541), as evidenced by the Product Review of Ambion's Poly(A)Pure mRNA Isolation Kit for the reasons set forth on pages 5 to 7 of the Office Action.

Chapkin et al. (USP 6,258,541) relate to a method for determining the expression of PKC isozymes.

For the following reasons, applicant respectfully disagrees with the position taken on page 5, lines 18 to 21 of the Office Action that Chapkin et al. teach a COX-2 detecting method for detecting colon cancer.

In Chapkin et al., wherein the effect of only PKC isozymes is confirmed, there is no specific disclosure or data supporting the usefulness of the COX-2 as a marker for colon cancer. There is no experimental data set forth in Chapkin et al. which demonstrates that COX-2 is detected in a fecal sample from a patient having colon cancer.

In column 6, line 14, Chapkin et al. do not list COX-2 alone, but describe only a combination of COX-1 and COX-2 as a risk marker for colon cancer. Chapkin et al. set forth approximately 45 different biomarkers in columns 5 to 6. There is no teaching in Chapkin et al. to lead one of ordinary skill in the art to choose COX-2 as a tumor marker out of the long list of biomarkers, and to utilize COX-2 in applicant's presently claimed

invention. There can be no anticipation where the reference is so broad that the likelihood of arriving at the claimed invention would be the same as discovering the combination of a safe by an inspection of its dials (Ex parte Garvey, 41 USPQ 583 (POBA 1939); Ex parte Starr and Beiswenger, 44 USPQ 545 (POBA 1938)), nor is anticipation made out by a hindsight selection based on an applicant's disclosure (In re Ruschig et al., 145 USPQ 274 (CCPA 1965)).

The disclosure of an anticipating prior art reference must be adequate to enable possession of applicant's claimed invention without undue experimentation (Elan Pharmaceuticals Inc. v. Mayo Foundation for Medical Education and Research, 68 USPQ 1373 (Fed. Cir. 2003); Inpax Laboratories v. Arentis Pharmaceuticals, 81 USPQ 2d 1001 (Fed. Cir. 2004)). Since Chapkin et al. do not disclose any testing with COX-2, it is respectfully submitted that Chapkin et al. is not an enabling disclosure with respect to the presently claimed invention.

In view of the above, it is respectfully submitted that Chapkin et al. do not teach COX-2 by itself as a marker for colon

cancer and do not teach or suggest applicants' presently claimed invention.

Withdrawal of the 35 USC 102 rejection is thus respectfully requested.

Obviousness Rejections Under 35 USC 103

Claims 15 and 16 were rejected under 35 USC 103 as being unpatentable over Chapkin et al. (USP 6,258,541) in view of Godfrey et al. (USP 7,101,663) for the reasons set forth in item no. 14 beginning at the bottom of page 7 and continuing to the top of page 9 of the Office Action.

It was admitted in the Office Action that Chapkin et al. do not teach wherein in applicant's step e) amplifying the cDNA from step d) is carried out by a nested PCR.

The Chapkin et al. reference was discussed hereinabove.

While Chapkin et al. describe the CEA as a tumor marker (column 6, line 7), CEA does not have both a high sensitivity and a high specificity compared to that of the COX-2 of the presently claimed invention (see Example 1 on pages 8 to 9 of the present

specification).

Godfrey et al. teach a nested PCR technique. The presently claimed invention is characterized by using COX-2 as a colon cancer marker.

For the reasons discussed hereinabove, it is respectfully submitted that a person of ordinary skill in the art would not consider to combine Chapkin et al. and Godfrey et al. to attempt to arrive at the presently claimed invention. Even assuming *arguendo* that Chapkin et al. and Godfrey et al. were combinable, for the reasons discussed hereinabove, a person of ordinary skill in the art would not arrive at the presently claimed invention based on the combination of the references.

Claims 5, 17 to 18, 20 and 23 were rejected under 35 USC 103 as being unpatentable over Alexander & Raicht, "Digestive Diseases and Sciences," Vol. 43, No. 12, pp. 2652-2658, (1998), as evidenced by Ultraspec TM-II RNA Isolation System Biotecx Bulletin No. 28, 1993 in view of Shattuck-Brandt et al., (1999), "Molecular Carcinogenesis," Vol. 24, pp. 177-187 and Lagerholm et al. (April 2001), "Gastroenterology," Vol. 120, No. 5, Suppl. 1,

Abstract, No. 16, Page A4 for the reasons set forth in item no. 15 beginning on page 9 and continuing to the top of page 14 of the Office Action.

Regarding applicant's claim 5, it was admitted in the Office Action that Alexander and Raicht do not teach the use of the marker COX-2 as a marker suitable for colon cancer detection.

Claims 15 and 16 were rejected under 35 USC 103 as being unpatentable over Alexander and Raicht, Shattuck-Brandt et al. and Lagerholm et al. and further in view of Godfrey et al. for the reasons set forth in item no. 16 on pages 14 to 16 of the Office Action.

Regarding applicant's claim 15, it was admitted in the Office Action that Alexander and Raicht, Shattuck-Brandt et al. and Lagerholm et al. do not teach wherein in applicant's step e) amplifying the cDNA from step d) is carried out with a nested PCR.

Godfrey et al. was discussed hereinabove.

The position was taken in the Office Action that Alexander and Raicht teach a method for detecting colon cancer "without

separating cell components from the feces" (page 10, lines 1 to 10 of the Office Action). However, based on the following reasons, Alexander and Raicht do not disclose such a feature.

Alexander and Raicht relate to a method of purifying total RNA from human stool using various phenol extraction steps. In the Alexander and Raicht method, the sample is first treated to separate cell components. As indicated by Alexander and Raicht, "in the first step, particulates and some bacteria are removed" (page 2653, Materials and Methods, second paragraph in the left-hand column, lines 4 to 5) (emphasis added). Alexander and Raicht further mention that the "slurry was decanted into sterile 1.5 ml Eppendorf tubes. These were centrifuged for 1 min at 4°C" (page 2653, Materials and Methods, fifth paragraph in the right-hand column) (emphasis added).

Alexander and Raicht therefore disclose a method of purifying total RNA from stool using phenol extraction after separating cell components by centrifugation.

In contrast with Alexander and Raicht, the presently claimed method relates to a method for preparing a sample to extract RNA

without separating cell components by centrifugation. Since it is well known that RNA is very sensitive to RNase, a person of ordinary skill in the art would have considered that extracting RNA directly from a biological sample containing an enormous amount of RNase would not be possible and that a step of separating cell components, prior to extracting RNA, is essential as disclosed in the prior art.

As disclosed in Example 2 on pages 10 to 11 of the present specification, when using the extraction method of Alexander and Raicht with a step of separating cell components, no PCR products were obtained. Therefore, the Alexander and Raicht method is not useful in detecting the COX-2 tumor marker.

Thus a person having ordinary skill in the art would not have a reasonable expectation to be able to detect the COX-2 using RT-PCR from RNA samples obtained from stool of colon cancer patients.

Further, whereas Alexander and Raicht teach some candidate genes useful for their method, e.g., GAPDH, etc., Alexander and Raicht are completely silent concerning using COX-2 as a tumor

marker (page 2657, second paragraph). Although it is known that COX-2 expression is markedly increased in human adenocarcinoma (see Shattuck-Brandt et al., page 178, left-hand column, second paragraph, lines 8 to 11), Alexander and Raicht do not describe using COX-2 as a tumor marker. Thus, Alexander and Raicht teach away from using COX-2 as a tumor marker.

Shattuck-Brandt et al. disclose that COX-2 expression is markedly increased in human adenocarcinoma. However, there is no description that the COX-2 can be detected from RNA samples obtained from human stool of colon cancer patients by using RT-PCR with both high sensitivity and high specificity.

A marker detected in a tissue sample does not always show high sensitivity and high specificity in a fecal sample. Nosho et al. (2005) (a copy of which was enclosed with the KANAOKA DECLARATION dated April 23, 2009) disclose that there is not much difference between the sensitivity of COX-2 and E1AF in a tissue sample, rather, E1AF is more sensitive than COX-2 (Nosho et al., Carcinogenesis, Vol. 26, pp. 892-899 (2005), a copy of which is enclosed). On the other hand, the DECLARATION UNDER 37 CFR 1.132

of Shigeru KANAOKA dated April 23, 2009 showed that the values of sensitivity and specificity of COX-2 in a fecal sample are greater than those of E1AF (see the second paragraph of the KANAOKA DECLARATION, page 4, which is reproduced in part as follows):

marker	sensitivity	specificity
COX-2	85.7%	100.0%
E1AF	27.5%	96.7%

Therefore, since the sensitivity and the specificity of a marker are varied based on the source of a sample, it cannot be said that a marker which is effective in a sample from a tissue is also effective in a sample from feces.

Lagerholm et al. disclose COX-2 expression in fecal colonocytes from patients with inflammatory bowel disease. However, it cannot be said that a marker effective in the detection of inflammatory bowel disease (IBD) is also effective in the detection of colon cancer. For example, calprotectin is detected in both colon cancer and IBD, and therefore the two

diseases cannot be distinguished by using calprotectin (Summerton et al., Eur. J. Gastroenterology & Hepatology, Vol. 14, No. 8, pp. 841-845 (2002), a copy of which is enclosed). It was also reported that calprotectin is a useful marker for IBD, however, it is not accurate for colon cancer (B.A. Lashner, Natl. Clin. Pract. Gastroenterology & Hepatology, Vol. 5, No. 1, pp. 16-17 (2008), a copy of which is enclosed; Am J. of Gastroenterology, 102, pp. 803-813 (2007), a copy of which is enclosed).

Thus, it cannot be said that a biomarker useful for the detection of IBD is useful for the detection of colon cancer.

Further, in Lagerholm et al., coloncytes were isolated from stool samples. As discussed above, the presently claimed method relates to a method for preparing a sample to extract RNA without separating cell components.

The differences between the presently claimed invention and the cited references are summarized in the following table:

	Presently Claimed Invention	Alexander and Raicht	Shattuck-Brandt et al.	Lagerholm et al.
biological sample	feces	feces	biopsy tissue sample	feces
cell component	in the presence	removed	-	removed
marker	COX-2	GAPDH	COX-2	COX-2
disease	colon cancer	colon cancer	colon cancer	inflammatory bowel disease
effect	both high sensitivity and high specificity	-	-	-

It is respectfully submitted that each of the references, i.e., Alexander and Raicht, Shattuck-Brandt et al. and Lagerholm et al., and even the combination thereof, does not provide a suggestion or teaching to a person of ordinary skill in the art to arrive at the presently claimed invention.

Further, the presently claimed invention affords surprising and unexpected results. As disclosed on page 9, lines 23 to 28 of the present specification, COX-2 was detected in 27 cases among the 30 colon cancer cases, but was not detected in 22 cases of the control group **(sensitivity: 90%, specificity: 100%)**.

On the other hand, CEA was detected in all cases among the 30 colon cancer cases and in 21 among 22 cases in the control group (page 9, lines 19 to 21 of the present specification).

Further, in a conventional immunological fecal occult blood test, 23 among 28 colon cancer cases and 3 among 22 control cases were positive **(sensitivity: 82.1%, specificity: 86.3%)**.

Thus, the method of the presently claimed invention has both high specificity and high sensitivity, which is surprising and unexpected from the references set forth in the Office Action.

The DECLARATION UNDER 37 CFR 1.132 of Shigeru KANAOKA dated April 23, 2009 and filed April 27, 2009 included comparative test data using an increased number of cases (from 30 to 70 patients with colorectal cancer and from 22 to 34 control subjects). It is respectfully submitted that the results of the April 23, 2009 KANAOKA DECLARATION show that the studied population consisted of 70 patients with CRC and 34 control subjects were enough to judge the effectiveness of the presently claimed method.

The results of the additional testing set forth in the April 23, 2009 KANAOKA DECLARATION are reproduced as follows:

Comparison of COX-2 Assay Between CEA Assay and Fecal Occult Blood Test

marker	sensitivity	specificity
COX-2	85.7% (60/70)	100.0% (34/34)
CEA	92.9% (65/70)	11.8% (4/34)
occult blood	73.5% (50/68)	82.4% (28/34)

The results for testing involving COX-2, E1AF and c-myc shown at the top of page 4 of the April 23, 2009 KANAOKA DECLARATION are reproduced as follows:

Comparison Among COX-2, E1AF and c-myc Assays

marker	sensitivity	specificity
COX-2	85.7% (60/70)	100.0% (34/34)
E1AF	27.5% (19/69)	96.7% (29/30)
c-myc	42.9% (15/35)	83.3% (20/24)

The above results show that the COX-2 assay had high sensitivity for detecting colorectal cancer, while maintaining high specificity compared with the other tested markers. None of

the other tested markers had both high sensitivity and high specificity.

The following statement concerning the April 23, 2009 KANAOKA DECLARATION ("additional data") was set forth in item no. 5 at the top of page 3 of the Office Action:

> "Examiner would like to point out that the additional data presented is found unpersuasive because the additional data presented is not commensurate with the scope of the claimed invention. Instant claims do not recite a particular level of sensitivity or specificity of detection of COX-2."

The undersigned had a telephone interview with Examiner Pande on November 4, 2009 regarding the preceding paragraph. During said telephone interview, the undersigned stated that the instant claims do not need to recite a particular level of sensitivity or specificity of the detection of COX-2 for the claims to be commensurate in scope with the showing in the April 23, 2009 KANAOKA DECLARATION. The undersigned informed the Examiner that the advantages inherent in a process which renders it patentable over the prior art need not be recited in applicant's claims (In re Estes, 164 USPQ 519 (CCPA 1970); In re

Merchant, 197 USPQ 785,788 (CCPA 1978)). The following is stated in 197 USPQ 788:

> "Finally, the solicitor repeats the objection voiced by the examiner that the declaration is irrelevant because the claims specify neither the unexpected result nor the 'features' that produce that result. We are aware of no law requiring that unexpected results relied upon for patentability be recited in the claims."

In view of the foregoing, the Examiner is respectfully requested to reconsider the April 23, 2009 KANAOKA DECLARATION.

It is respectfully submitted that applicant's presently claimed invention patentably distinguishes over the references singly or combined in the manner set forth in the Office Action.

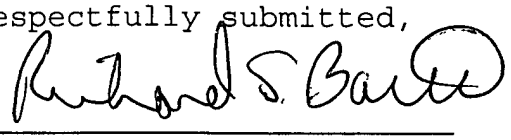
Withdrawal of each of the 35 USC 103 rejections is thus respectfully requested.

Reconsideration is requested. Allowance is solicited.

If the Examiner has any comments, questions, objections or recommendations, the Examiner is invited to telephone the

undersigned at the telephone number given below for prompt action.

Respectfully submitted,



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(2) a copy of Carcinogenesis, Vol. 26, pp. 892-899 (2005)

(3) a copy of Eur. J. Gastroenterology & Hepatology, Vol. 14, No. 8, pp. 841-845 (2002)

(4) a copy of Natl. Clin. Pract. Gastroenterology & Hepatology, Vol. 5, No. 1, pp. 16-17 (2008)

(5) a copy of Am. J. of Gastroenterology, 102, pp. 803-813 (2007)

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Association of Ets-related transcriptional factor E1AF expression with overexpression of matrix metalloproteinases, COX-2 and iNOS in the early stage of colorectal carcinogenesis

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It is now becoming clear that matrix metalloproteinases (MMPs) play a key role in tumor development and growth. MMPs are overexpressed in a variety of premalignant tumor tissues, including colorectal adenoma. Little is known about the mechanisms underlying the overexpression of MMPs in adenoma tissues. E1AF, an Ets family transcriptional factor, has been shown to play an important role in the expression of MMPs and cyclooxygenase-2 (COX-2) in advanced colorectal cancers. The aim of this study was to examine the E1AF expression and determine whether it is correlated with the expression of MMPs, COX-2 and inducible nitric oxide synthase (iNOS) in human colorectal adenoma and submucosal cancer (pT1). Using the semi-quantitative RT–PCR, 90 colorectal tumors, including 63 adenomas and 27 cancers (pT1), were analyzed for the expression of E1AF, MMPs, COX-2 and iNOS. Immunohistochemical analysis and *in vitro* transfection assays were also performed. E1AF mRNA was detected in 43 (47.8%) of the 90 colorectal tumors. E1AF overexpression was significantly correlated with histopathology. E1AF expression was correlated significantly with the expression of MMP-1 and MMP-7. Overexpression of COX-2 and iNOS mRNA expression was observed in 42.2% and 66.7% of the 90 colorectal tumors, respectively. COX-2 was correlated significantly with size, gender, histopathology and E1AF. iNOS was correlated significantly with size, histopathology, E1AF and COX-2. The correlation of E1AF expression with COX-2 and iNOS expression was also demonstrated by immunohistochemistry. Northern blot analysis of transfectants showed the effect of E1AF on COX-2 expression as well as iNOS on E1AF/COX-2 expression in colon cancer cell lines. The results suggest that E1AF, in conjunction with the expression of MMP-1, MMP-7, COX-2 and iNOS, plays an important role in the early stage of colorectal carcinogenesis.

Abbreviations: COX-2, cyclooxygenase-2; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; NO, nitric oxide; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction; SMT, S-methylisothiourea sulfate; SSC, standard saline citrate.

†The first two authors contributed equally to this work.

Introduction

Colorectal cancer is one of the most common human malignancies in the world. Although alternative pathways exist, it is accepted that most colorectal cancers arise in pre-existing adenomas (1).

Degradation of the extracellular matrix (ECM) mediated by matrix metalloproteinases (MMPs) is crucial during tumor invasion and metastasis (2–8). Until recently, the role of MMPs has been associated primarily with tumor invasion and metastasis. It is now becoming clear that MMPs also play a key role in tumor development and growth (2–8). Several lines of evidence indicate that MMPs regulate cell growth and survival. MMPs directly participate in the generation of signals that induce the proliferation of tumor cells by activating the cell surface growth factor precursors, releasing and activating latent growth factors sequestered in the ECM, and altering the structure of essential ECM components (2–11). Indeed, MMPs are overexpressed in a variety of premalignant tumor tissues, including colorectal adenoma tissues (12–18).

However, despite the fact that several studies have shown that MMPs play important roles in the early stage of colorectal carcinogenesis, little is known about the mechanisms underlying the overexpression of MMPs in adenoma tissues. We recently reported that E1AF (human PEA3/ETV4), an Ets family transcriptional factor, plays a key role in the progression of colorectal cancer (19). Interestingly, the expression of E1AF was closely correlated with the expression of MMP-1 and MMP-7 in advanced colorectal cancer tissues.

Interestingly, it has been reported that E1AF is one of the potent activators of cyclooxygenase-2 (COX-2) transcription (20). Increasing mounting evidence indicates that COX-2 plays an important role in the early and late stages of colorectal carcinogenesis (21–26). COX-2 is overexpressed in 80–90% of colorectal cancer tissues and in 40–50% of premalignant adenoma tissues (16,17).

Inducible nitric oxide synthase (iNOS) has been reported to play a crucial role in the development of cancer by promoting angiogenesis (27). Cianchi *et al.* (28,29) reported that COX-2 and iNOS upregulated VEGF, which is one of the most important proangiogenic factors, in human colorectal cancer. Moreover, nitric oxide (NO), generated by iNOS, reportedly stimulates E1AF to increase the COX-2 expression in colorectal cancer (30). In addition, NO has been shown to augment the synergistic interaction between E1AF and its transcription coactivator CBP/p300, resulting in the facilitation of COX-2 induction (30).

Thus, it seems important to clarify whether E1AF, MMPs, COX-2, and iNOS are overexpressed concomitantly in the early stage of colorectal carcinogenesis. If this is the case, as a transcriptional factor, E1AF may play an important role in the regulation of multiple genes involved in tumour promotion. In an attempt to address these issues, we investigated the expression of E1AF and other Ets-related transcriptional factors in 90 colorectal tumor tissues, including 63 adenoma

tissues and 27 cancer tissues with submucosal invasion (pT1), by using the semi-quantitative RT–PCR, with respect to clinicopathological characteristics and the expression of MMPs, COX-2 and iNOS. Considering the limits of semi-quantitative RT–PCR analysis, we further analyzed the expression of E1AF, COX-2, and iNOS immunohistochemically. *In vitro* transfection assays were also performed.

Materials and methods

Patients and tissue samples

Ninety paired specimens of colorectal tumor and non-tumor tissues were obtained by polypectomy or surgical treatment. These tumor samples consisted of 63 adenomas and 27 adenocarcinomas with submucosal invasion (pT1 in the TNM classification of the Union Internationale Contre le Cancer). Each tissue specimen was divided into two pieces. For total RNA extraction, one sample was immediately frozen in liquid nitrogen at the time of endoscopy or surgery and stored at −80°C until extraction. The other sample was processed for pathological examination using hematoxylin and eosin staining for the evaluation of the tumor cell content. The histopathological features of the specimens were classified according to the TNM classification system. Locations of the colorectal tumors were divided into proximal colon (cecum, ascending and transverse colon) and distal colon (descending and signoid colon and rectum). Macroscopic types were divided into protruded type (height of tumor ≥3 mm) and flat type (height of tumor <3 mm). The clinicopathological characteristics of colorectal tumors are shown in Table I. An informed consent was obtained from each subject and the institutional review committee approved this study.

Semi-quantitative RT–PCR

Total RNA was extracted from specimens using the acid guanidinum thiocyanate–phenol–chloroform extraction method and treated with DNase I. cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, San Diego, CA) with random hexamers. PCR was performed using primers specific for each target gene and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes in duplex PCR (12). GAPDH served as an internal control of the reaction. The reactions were controlled without reverse transcriptase. Results were analyzed using a multi-image analyzer (Bio-Rad, Richmond, CA). The levels of gene transcripts were quantified as the ratio of the intensity of the target gene to the intensity of GAPDH. Overexpression was judged when the target gene expression in the tumor samples was at least three times higher than that in the corresponding normal sample. To perform semi-quantitative RT–PCR, the ranges of linear amplification for the target gene and for the GAPDH genes were studied. The optimal number of PCR cycles and the mixing ratio of primers were determined. The primers used were 5′-GCCCATTTCATTGCCTGGAC-3′ and 5′-GACTTGCCATTTCTCCACTTTCC-3′ for E1AF, 5′-AGCAGCATGGATGGATTTTAT-3′ and 5′-CTCCTGCTTAAAGCCTTGTGGTGG-3′ for ER81, 5′-TTATGGTCCCAGGGAAATCTCGAT-3′ and 5′-TGGCAGGGTTCAGACAGTTGTCTC-3′ for ERM, 5′-GGGTAGCGACTTCTTGTTTG-3′ and 5′-GTTAATGGAGTCAACCCAGC-3′ for Ets-1, 5′-GCCTCAATAAGCCAACCATGTC-3′ 5′-TCAATCCTGCCTTTCCTGGGTC-3′ for Ets-2, AGATGTGGAGTGCCTGATGT-3′ and 5′-AGCTAGGGTACATCAAAGCC-3′ for MMP-1, 5′-AGAGGTGAC TCCACTCACAT-3′ and 5′-GGTCTGTGAGTGAGTGATAG-3′ for MMP-3, 5′-TCTTTGGCCTACCTATAACTGG-3′ and 5′-CTAGACTGCTACCATCCGTC-3′ for MMP-7, 5′-A CGGGCTCCTGGCACACG-3′ and 5′-CGTCCCGGGTGTAGAGTC-3′ for MMP-9, 5′-TTCAAATGAGATTGTGGGAAAAT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ for COX-2, 5′-GCCTCGCTCTGGAAAGA-3′ and 5′-TCCATGCAGACAACCTT-3′ for iNOS, 5′-GGCGTCTTCACCACCATGGAG-3′ and 5′-AAGTTGTCATGGATGACCTTGGC-3′ for GAPDH.

Immunohistochemistry

Sixty formalin-fixed, paraffin-embedded colorectal tumor specimens were obtained from patients who had undergone polypectomy or surgical treatment. These tumor samples consisted of 42 adenomas and 18 adenocarcinomas with submucosal invasion. Sections of formalin-fixed and paraffin-embedded tissue of 5 μm thickness were de-waxed in xylene and rehydrated in alcohol. The sections were then heated to 105°C in a target retrieval solution (DakoCytomation, Carpinteria, CA) in an autoclave for 10 min, for antigen retrieval. Endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide in methanol for 5 min. After being rinsed twice in phosphate-buffered saline (PBS), the sections were incubated for 18 h at 4°C with an anti-human PEA3 mouse monoclonal antibody (10 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human COX-2 mouse monoclonal antibody (10 μg/ml, ZYMED Laboratories, San Francisco, CA) or anti-human iNOS mouse monoclonal antibody (10 μg/ml, Transduction Laboratories, Lexington, NY). The antibodies were diluted in antibody diluent with background reducing components (0.05 mol/l Tris–HCl buffer containing 0.1% Tween and 0.015 mol/l sodium azide) (DakoCytomation, Glostrup, Denmark). Normal mouse immunoglobulins were substituted for each primary antibody as negative controls. After washing three times in PBS, the sections were treated with biotinylated anti-mouse immunoglobulin (DakoCytomation) for 10 min and then with horseradish peroxidase–avidin complex, diluted as recommended by the manufacturer, for 10 min. The slides were then washed in PBS and developed in 0.05 M Tris–HCl (pH 7.5) containing 0.6 mg/ml of 3,3′ diaminobenzidine at room temperature. The sections were counterstained in Mayer's hematoxylin and mounted. The sections were examined microscopically by two well-trained pathologists who were blinded to the clinicopathological characteristics. Nuclear expression of E1AF and cytoplasmic expression of COX-2 and iNOS were defined as positive when an immunoreactivity was observed in > 10% of tumor cells.

DNA transfection

The human colon cancer cell line RCM-1 was purchased from Japanese Collection of Research Bioresources (Tokyo). Cells were cultured in RPMI-1640 containing 10% fetal bovine serum. A full-length cDNA encoding human iNOS was cloned into a eukaryotic expression vector, pcDNA3.1 (+) (Invitrogen, San Diego, CA) under the control of the cytomegalovirus promoter in a sense orientation and the vector was designated as pcDNA3.1/iNOS. pcDNA3.1/iNOS or pcDNA3.1 was transfected into RCM-1 cells using SuperFect transfection reagent (Quiagen, Hilden, Germany), following the manufacturer's protocol. After a few weeks of G418 selection, individual colonies were selected and expanded for further analyses. Transfectants containing the vector plasmid pcDNA3.1 alone were used as controls. iNOS inhibitor *S*-methylisothiourea sulfate (SMT) was purchased from Calbiochem (San Diego, CA). Some transfectants were treated with 50 μM SMT for 14 h.

Northern blot analysis

Total RNA was prepared from cells using the acid guanidinium thiocyanate–phenol–chloroform extraction method, followed by a treatment with deoxyribonuclease I. Ten micrograms of total RNA were electrophoresed on a 1% denaturing agarose gel and transferred onto a nitrocellulose membrane. The membrane was hybridized with a complementary DNA for E1AF, COX-2 or iNOS labeled using the random primer method in 50% formamide/5× Denhardt's solution/3× standard saline citrate (SSC)/100 μg/ml salmon sperm DNA/1% SDS at 42°C overnight. The membrane was then washed twice in 2× SSC/0.1% SDS at room temperature for 10 min and three times in 0.1× SSC/0.1% SDS at 55°C for 15 min. After washing, the membrane was exposed to X-ray films at −70°C. The membrane was then stripped and reprobed with a β-actin complementary DNA probe to control for the quantity of loading and integrity of total RNA in each lane.

Statistical analysis

Expression of each target gene was assessed for associations with clinicopathological characteristics using the following statistical tests: Mann–Whitney *U*-test for age, size and average tumor–normal expression ratios, and the chi-square two-tailed test or Fisher's exact test for the remaining parameters.

Results

E1AF mRNA expression in colorectal tumor tissues

To perform semi-quantitative RT–PCR analysis, the ranges of linear amplification for each target gene and for the control GAPDH gene were examined. The optimal number of PCR cycles and optimal mixing ratios of primers were determined. The expression of E1AF mRNA in 90 colorectal tumor tissues was examined. Figure 1 shows the representative results of RT–PCR for E1AF. E1AF mRNA expression was detected in 43 (47.8%) of the 90 colorectal tumor tissues but was undetectable in adjacent non-tumor tissues. The relationships between E1AF expression and clinicopathological characteristics are shown in Table I. E1AF mRNA expression was correlated significantly with histopathology ($P = 0.0188$). There was no correlation of E1AF mRNA expression with age, size, gender, location or macroscopic type. The average tumor–normal expression ratio was significantly higher in pT1 cancer than in adenoma ($P = 0.0006$). Expression of ER81 and ERM was

Table I. Clinicopathological characteristics and mRNA expression profiles in 90 colorectal tumor tissues. Each row is a colorectal adenoma ($n = 63$) or pT1 cancer ($n = 27$). Black rectangles indicate each mRNA expression as positive. Gender (M, male; F, female), location (D, distal; P, proximal), macroscopic type (P, protruded; F, flat).

Adenoma

Case	Age	Size	Gender	Location	Macroscopic type
No. 1	74	8	F	P	P
2	47	3	M	D	F
3	49	5	M	D	P
4	26	6	M	D	P
5	69	6	M	D	P
6	55	6	F	D	P
7	55	5	F	D	F
8	55	4	F	P	P
9	68	13	F	P	P
10	68	13	F	P	P
11	68	13	F	P	P
12	74	10	M	P	F
13	59	20	M	D	P
14	68	9	F	D	F
15	78	8	F	D	P
16	78	6	F	D	P
17	77	40	M	D	F
18	42	3	F	D	F
19	60	20	M	D	P
20	60	15	M	D	P
21	60	6	M	D	P
22	60	10	M	P	P
23	60	6	M	P	P
24	66	4	M	P	F
25	27	3	F	D	F
26	65	6	M	D	F
27	65	40	M	D	F
28	59	15	M	P	P
29	66	12	M	P	P
30	66	12	M	D	P
31	66	12	M	D	P
32	66	8	M	P	F
33	74	9	M	D	P
34	74	4	M	P	F
35	74	7	M	D	F
36	74	6	M	D	F
37	74	15	M	P	F
38	66	6	M	D	P
39	59	40	M	D	P
40	81	3	F	D	P
41	81	15	F	D	F
42	81	40	F	D	F
43	74	14	F	P	F
44	72	15	F	P	F
45	50	10	F	D	P
46	59	10	M	D	P
47	66	8	M	P	F
48	74	5	M	P	F
49	74	7	M	P	P
50	78	15	M	D	P
51	66	6	F	D	P
52	74	13	M	P	P
53	74	8	M	P	P
54	74	5	M	P	P
55	60	6	F	D	P
56	60	8	F	D	P
57	61	10	F	D	F
58	55	20	F	D	F
59	65	12	F	P	F
60	62	6	F	D	P
61	62	5	F	D	P
62	62	10	F	D	P
63	60	10	F	D	P

Cancer (pT1)

Case	Age	Size	Gender	Location	Macroscopic type
No. 1	78	14	F	D	P
2	72	10	M	D	P
3	72	15	M	D	P
4	55	10	F	D	P
5	63	12	M	D	P
6	68	45	F	P	P
7	62	35	F	P	F
8	74	5	M	P	F
9	59	20	M	D	P
10	81	20	F	D	P
11	81	20	F	P	P
12	66	22	M	P	P
13	70	17	F	D	P
14	68	23	F	D	P
15	69	19	M	D	P
16	67	20	M	D	P
17	70	19	F	D	P
18	73	22	M	P	P
19	67	15	M	P	P
20	60	18	M	D	P
21	61	22	F	P	P
22	60	21	M	D	P
23	69	22	M	D	F
24	74	22	F	D	F
25	66	8	M	D	F
26	66	20	F	D	F
27	55	19	F	P	F

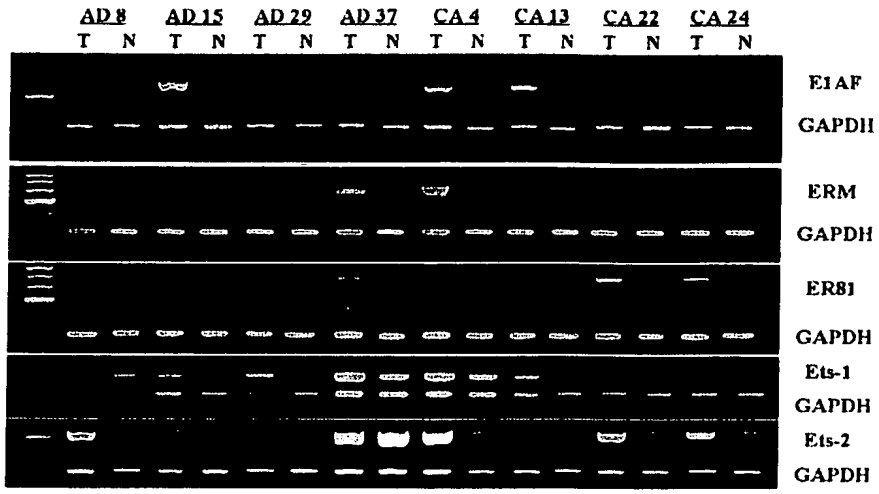

Fig. 1. RT–PCR analysis of mRNA expression for E1AF, ERM, ER81, Ets-1 and Ets-2 in colorectal tumor tissues. T and N, matched samples from tumor and non-tumor tissue, respectively. Cases 1–4 are colorectal adenomas and cases 5–8 are colorectal carcinomas (pT1).

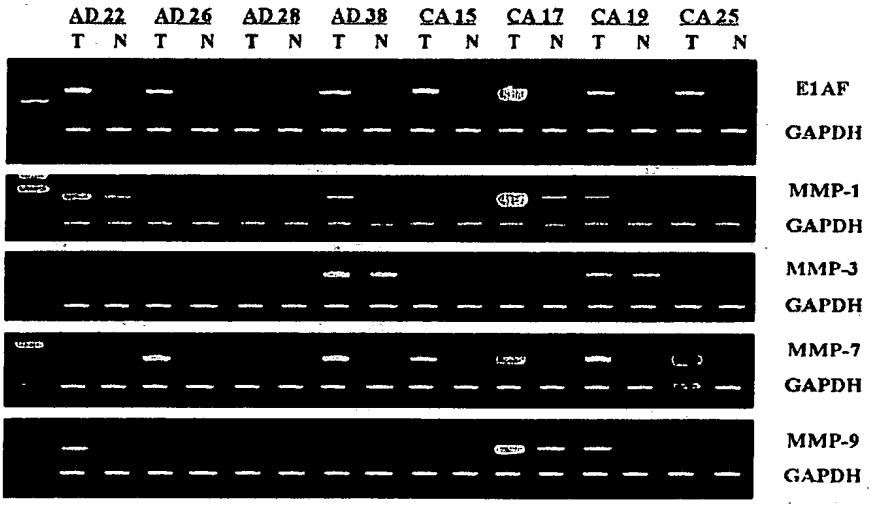

Fig. 2. RT–PCR analysis of mRNA expression for E1AF, MMP-1, MMP-3, MMP-7 and MMP-9 in colorectal tumor tissues. T and N, matched samples from tumor and non-tumor tissue, respectively. Cases 1–4 are colorectal adenomas and cases 5–8 are colorectal carcinomas (pT1).

faintly detected in non-tumor tissues (Figure 1). Overexpression of ER81 and ERM mRNA was observed in 20.0% and 16.7% of the 90 colorectal tumor tissues, respectively, but the expression was not correlated significantly with any of the clinicopathological characteristics (data not shown). The expression patterns of the three related genes, E1AF, ER81 and ERM were not significantly correlated with each other. For comparison, we also analyzed the expression of Ets-1 and Ets-2 in colorectal tumor tissues (Figure 1). Overexpression of Ets-1 and Ets-2 mRNA was observed in 38.9% and 60.0% of the 90 colorectal tumor tissues, respectively, but the expression was not correlated significantly with any of the clinicopathological characteristics (data not shown).

Expression of MMPs and their relationships with E1AF expression

The expression of MMPs mRNA was examined in 90 colorectal tumor tissues. Figure 2 shows the representative results of RT–PCR for E1AF, MMP-1, MMP-3, MMP-7 and MMP-9. Expression of MMP-7 was undetectable or only faintly detected in adjacent non-tumor tissues. Overexpression of MMP-1, MMP-3, MMP-7 and MMP-9 mRNA was observed in 37.8%, 0%, 77.8% and 16.7% of the 90 colorectal tumor tissues, respectively.

The relationships between the expression of MMPs and clinicopathological characteristics are shown in Table I. The expression of MMP-1 was correlated significantly with size ($P = 0.0035$). MMP-7 mRNA expression was correlated significantly with size ($P = 0.0008$), age ($P = 0.0150$), location ($P = 0.0394$) and histopathology ($P = 0.0057$). The expression of MMP-9 was correlated significantly with size ($P = 0.0003$) and histopathology ($P < 0.0001$). When only adenoma tissues were considered, the correlation of MMP-1 with size ($P = 0.0013$) and that of MMP-7 expression with size ($P = 0.0170$) and age ($P = 0.0354$) were still significant. Average tumor–normal expression ratios of MMP-1, MMP-7 and MMP-9 were

significantly higher in pT1 cancer than in adenoma ($P = 0.0490$, $P = 0.0310$ and $P < 0.0001$, respectively). Among the expression of MMPs analyzed, E1AF expression was correlated significantly with the expression of MMP-1 and MMP-7 ($P = 0.0385$ and $P = 0.0048$, respectively, Table II). When only adenoma tissues were considered, the correlation between E1AF and MMP-7 expression was still significant ($P = 0.0109$).

COX-2 and iNOS mRNA expression in colorectal tumor tissues

Figure 3 shows the representative results of RT-PCR for COX-2 and iNOS. COX-2 mRNA expression was detected in 38 (42.2%) of the 90 colorectal tumor tissues but was undetectable or only faintly detected in adjacent non-tumor tissues. The relationships between the COX-2 overexpression and clinicopathological characteristics are shown in Table I. COX-2 expression was correlated significantly with size ($P = 0.0002$), gender ($P = 0.0042$), histopathology ($P = 0.0004$) and E1AF expression ($P < 0.0001$; Table III). Average tumor–normal expression ratios were significantly higher in pT1 cancer than in adenoma ($P < 0.0001$). When only adenoma tissues were considered, the correlation of COX-2 expression with size, gender and E1AF expression was still significant ($P = 0.0443$, $P = 0.0118$ and $P = 0.0003$, respectively).

iNOS mRNA expression was detected in 60 (66.7%) of the 90 colorectal tumor tissues but was undetectable or only faintly detected in adjacent non-tumor tissues. The relationships between iNOS overexpression and clinicopathological characteristics are shown in Table I. iNOS expression was correlated significantly with size ($P < 0.0001$), histopathology ($P = 0.0006$), COX-2 expression ($P < 0.0001$) and E1AF expression ($P = 0.0002$; Table III). Average tumor–normal expression ratios were significantly higher in pT1 cancer than in adenoma ($P < 0.0001$). When only adenoma tissues were considered, the correlation of iNOS expression with size, COX-2 and E1AF expression was still significant ($P = 0.0047$, $P = 0.0004$ and $P = 0.0081$, respectively).

Immunohistochemical expression of E1AF, COX-2 and iNOS in colorectal tumor tissues

Figure 4 shows the representative the results of immunohistochemical expression of E1AF, COX-2 and iNOS in a patient (Case no. 3 in cancer group). Immunohistochemical expression of E1AF, COX-2 and iNOS was positive in 24 (40.0%), 28 (46.7%) and 37 (61.7%) of the 60 tumors, respectively. E1AF expression was correlated significantly with COX-2 expression and iNOS expression (18 COX-2-positive/24 E1AF-positive versus 10 COX-2-positive/36 E1AF-negative, $P = 0.0003$ and 19 iNOS-positive/24 E1AF-positive versus 18 iNOS-positive/36 E1AF-negative, $P = 0.0013$). COX-2 expression was correlated significantly with iNOS expression (20 iNOS-positive/28 COX-2-positive versus 17 iNOS-positive/32 COX-2-negative, $P = 0.0023$).

Table II. Overexpression of MMPs mRNA expression and its relationship with E1AF mRNA expression

	E1AF mRNA expression		*P*-value
	Positive $n = 43$	Negative $n = 47$	
MMP-1 mRNA expression			
Present	21	13	
Absent	22	34	0.0385
MMP-7 mRNA expression			
Present	39	31	
Absent	4	16	0.0048
MMP-9 mRNA expression			
Present	10	5	
Absent	33	42	0.1086

Table III. Overexpression of COX-2 and iNOS mRNA expression and its relationship with E1AF mRNA expression

	E1AF mRNA expression		*P*-value
	Positive $n = 43$	Negative $n = 47$	
COX2 mRNA expression			
Present	29	9	
Absent	14	38	<0.0001
iNOSmRNA expression			
Present	37	23	
Absent	6	24	0.0002

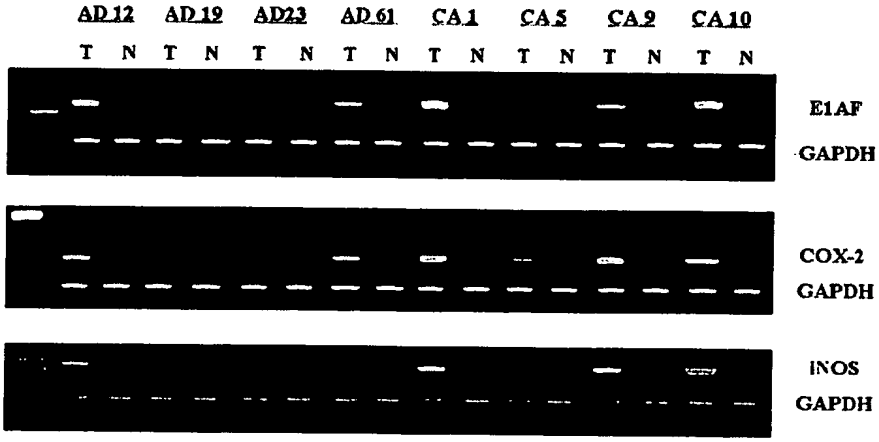

Fig. 3. RT-PCR analysis of mRNA expression for E1AF, COX-2 and iNOS in colorectal tumor tissues. T and N, matched samples from tumor and non-tumor tissue, respectively. Cases 1–4 are colorectal adenomas and cases 5–8 are colorectal carcinomas (pT1).

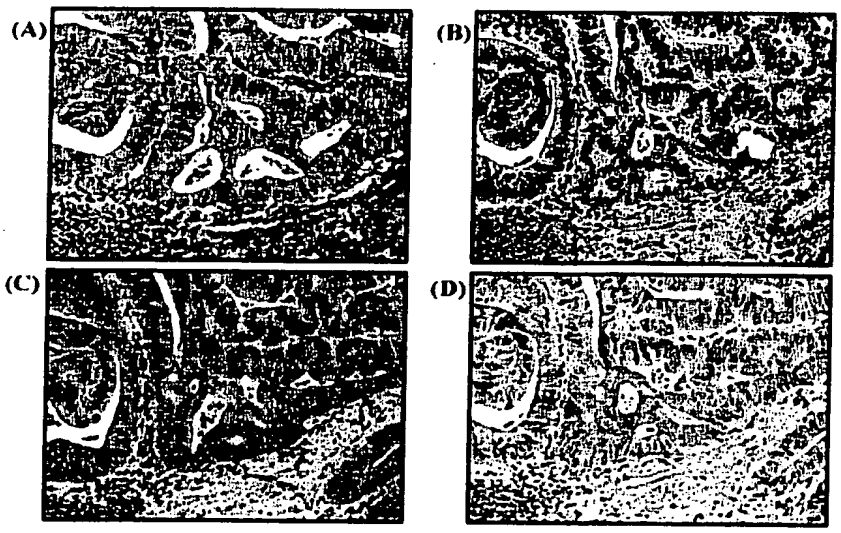

Fig. 4. Hematoxylin–eosin staining (A) and immunohistochemical analysis for E1AF (B), COX-2 (C) and iNOS (D) in serial sections of colon cancer tissues (Case no. 3 in cancer group). (A) Hematoxylin–eosin stained section. (B) Nuclear expression of E1AF in cancer cells. (C) Cytoplasmic expression of COX-2 in cancer cells. (D) Cytoplasmic expression of iNOS in cancer cells. (A–D) Original magnification ×200.

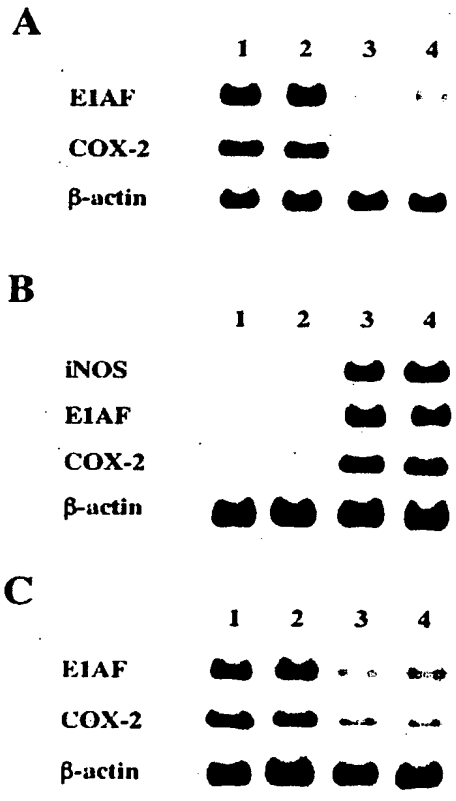

Fig. 5. The effect of E1AF on COX-2 expression as well as iNOS on E1AF/COX-2 expression in colon cancer cell lines. (A) Northern blot analysis of parental HT-29 cells and transfectants. Lane 1, parental HT-29 cells; 2, mock-transfected HT-29; 3, antisense E1AF-transfected HT-29 (HT-AS-3); 4, HT-AS-7. (B) Northern blot analysis of parental RCM-1 cells and transfectants. Lane 1, parental RCM-1 cells; 2, mock-transfected RCM-1; 3, iNOS-transfected RCM-1 (RCM/iNOS-2); 4, RCM/iNOS-6. (C) Northern blot analysis of iNOS-transfectants without or with treatment of iNOS inhibitor SMT. Lane 1, RCM/iNOS-2; 2, RCM/iNOS-6; 3, RCM/iNOS-2 treated with SMT; 4, RCM/iNOS-6 treated with SMT

Effect of E1AF on COX-2 expression as well as iNOS on E1AF/COX-2 expression in colon cancer cell lines

We previously cloned antisense E1AF-transfected HT-29 cells and confirmed a considerable reduction in the amount of E1AF mRNA in the HT-29-derived clones HT AS-3 and HT AS-7 (19). Therefore, we analyzed the COX-2 expression by northern blot analysis in parental HT-29 cells, mock-transfected HT-29 cells, and HT AS-3 and HT AS-7 clones. A considerable reduction in the amount of COX-2 mRNA was observed in the HT AS-3 and HT AS-7 clones (Figure 5A).

RCM-1 cells were stably transfected with a cytomegalovirus-based vector that carried the iNOS cDNA in a sense orientation. After G418 selection, 10 different clones were analyzed for iNOS mRNA by northern blot analysis. A considerable amount of iNOS mRNA was observed in the RCM-1-derived clones RCM/iNOS-2 and RCM/iNOS-6, and the expression of E1AF and COX-2 was significantly upregulated in these clones (Figure 5B). iNOS inhibitor SMT suppressed the effect of iNOS in augmenting the expression of E1AF and COX-2 (Figure 5C).

Discussion

The issue that we addressed in this study was the expression of E1AF and its relationship with the expression of MMPs, COX-2 and iNOS in the early stage of colorectal carcinogenesis. The reason why we chose pT1 cancer is that it represents the early stage of colorectal cancer.

E1AF mRNA was detected in 47.8% of the 90 colorectal tumor tissues but not in adjacent non-tumor tissues, suggesting that the tumor-associated expression of E1AF occurs in the early stage of colorectal carcinogenesis. The frequency of E1AF mRNA expression was significantly higher in pT1 cancer (66.7%) than in adenoma (39.7%). It has been shown that an increase in both the amount and activity of E1AF is needed to assure a high E1AF target gene expression (31). In this aspect, it is interesting that the average tumor–normal expression ratio of E1AF was significantly higher in pT1 cancer

than in adenoma. It is possible that an initiating genetic event leads to the upregulation of the transcriptional activity of E1AF and that transcriptionally activated E1AF, in turn, stimulates the expression of its target genes, including the E1AF gene itself (31).

As for the expression of MMPs, overexpression of MMP-1, MMP-3, MMP-7 and MMP-9 mRNA was observed in 37.8%, 0%, 77.8% and 16.7% of the 90 colorectal tumor tissues, respectively. The expression of MMP-1 was correlated significantly with size. MMP-7 mRNA expression was correlated significantly with size and histopathology. The expression of MMP-9 was correlated significantly with size and histopathology. Average tumor-normal expression ratios of MMP-1, MMP-7 and MMP-9 were significantly higher in pT1 cancer than in adenoma. These results suggest that MMP-1, MMP-7 and MMP-9 are involved in tumor growth and/or early invasion of tumor cells.

The expression of E1AF mRNA was correlated significantly with expression of MMP-1 and MMP-7 in the early stage of colorectal carcinogenesis. Interestingly, a concomitant expression of E1AF and MMP-7 mRNA has been shown in intestinal adenoma tissues in Min mice (32). These results further support the notion that E1AF plays an important role in the induction of MMP-7 expression in colorectal adenoma tissues.

The expression of E1AF mRNA was also correlated significantly with the expression of COX-2 in colorectal tumor tissues. The association was further substantiated by immunohistochemistry. Moreover, a northern blot analysis of antisense E1AF transfectants also showed the effect of E1AF on COX-2 expression in colon cancer cell lines. Although COX-2 expression is regulated by both transcriptional and post-transcriptional mechanisms, transcriptional regulation may play a more decisive role in COX-2 expression in human colon carcinoma cells (26,33,34). It has been shown that while β-catenin only weakly activates the COX-2 promoter, E1AF is a potent activator of COX-2 transcription (20). It has been shown that the expression of MMP-7 and COX-2 is responsive to Wnt signaling and that their expression is regulated by E1AF in colorectal cancer cell lines (20). Thus, our results suggest that E1AF plays an important role in the induction of MMP-1, MMP-7 and COX-2 in the early stage of colorectal carcinogenesis. The overall frequency of nodal metastasis of pT1 colorectal cancer has been reported to be ~10%. Liver metastasis is also rare. To clarify whether the COX-2 expression is associated with a metastasis of early invasive colorectal cancer, studies on a large number of pT1 cancer tissues are needed. Because transcriptional and translational regulation of COX-2 is complex (20,26,33), additional studies are required to clarify the complexity of COX-2 regulation in the early stage of colorectal carcinogenesis.

As for the expression of iNOS, Yagihashi *et al.* (35) previously reported a 5-fold increase in the mRNA level in 50% of six colorectal cancer tissues. In the current study, iNOS mRNA expression was detected in 66.7% of the 90 colorectal tumor tissues but was undetectable or only faintly detected in adjacent non-tumor tissues. iNOS mRNA overexpression was correlated significantly with size and histopathology. Average tumor-normal expression ratio was significantly higher in pT1 cancer than in adenoma. These results suggest that iNOS contributes to the tumor growth and early invasion of tumor cells. Interestingly, the expression of iNOS mRNA was correlated significantly with the expression of E1AF and COX-2. The associations were further substantiated by immunohistochemistry. Moreover, northen blot analysis of iNOS transfectants with or without iNOS inhibitor also showed the effect of iNOS on E1AF/COX-2 expression in colon cancer cell lines. It has been thought that NO, generated by iNOS, stimulates the activity of MMPs, which in turn, causes the degradation of E-cadherin and the relocalization of β-catenin to the cytoplasm and nucleus, and activates the β-catenin–TCF/LEF signaling pathway (30). β-catenin–TCF/LEF reportedly activates E1AF, which stimulates the COX-2 activity (30). In addition, NO has been shown to augment the synergistic interaction between E1AF and its transcription coactivator CBP/p300, resulting in the facilitation of COX-2 induction (30). Our results support those of the previous *in vitro* study (30) and further suggest that iNOS, in conjunction with E1AF and COX-2, plays an important role in the early colorectal carcinogenesis.

Thus, the most important aspect of this study is the early appearance of gene products of the E1AF, MMPs, COX-2 and iNOS in colorectal tumor tissue samples. As a tumor-associated transcriptional factor, E1AF appears to play an important role in the regulation of multiple genes involved in tumour promotion. The results of *in vitro* studies also support this notion.

All the biopsy samples were obtained from the surface of the tumor in this study. Therefore, E1AF mRNA expression is derived from the tumor cells in the lamina propria mucosae. In addition, using the immunohistochemical method, staining of E1AF was observed not only at the invasive front but also in the upper part of the muscularis mucosae (19). Accordingly, it is thought that a tumor in which E1AF is overexpressed, already has malignant potential before it invades the submucosa. Therefore, analysis of the E1AF expression in colorectal adenoma tissues obtained by biopsy could be useful for the prediction of malignant potential, preoperatively. The E1AF-MMP1-MMP-7-COX-2-iNOS axis could be a potent therapeutic target in patients with a colorectal tumor. Therapeutic agents that inhibit the expression or function of E1AF or its target genes may prove efficacious or might complement agents that compromise MMP, COX-2 and iNOS activities in the treatment of colorectal tumors and other tumors characterized by an E1AF overexpression (36).

Acknowledgements

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Faecal calprotectin: a marker of inflammation throughout the intestinal tract

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See leading article pp. 823–825

Objective **To assess the potential of measuring the calcium-binding protein calprotectin in faeces as a method of screening for alimentary inflammation and neoplasia.**

Setting **Hospital day services unit for endoscopy and faecal analysis in the clinical biochemistry department.**

Participants **Consented patients attending for routine endoscopy were requested to provide faeces. Seventeen of the initial 30 patients provided faeces before and 1 week after endoscopy. After this, 116 patients for planned endoscopy provided faeces before endoscopy. The group comprised 43 patients with upper-gastrointestinal lesions, seven patients with inflammatory bowel disease, seven patients with irritable bowel syndrome, 31 patients with colonic disorders, and 28 normal people. A final 18 patients with known inflammatory bowel disease (seven patients), gastric carcinoma (one patient), colorectal cancer (eight patients) and colorectal adenoma (two patients) had faeces analysed.**

Method **Faeces were analysed by the Nycotest PhiCal enzyme-linked immunosorbent assay (ELISA) (Nycomed, Oslo, Norway), and the final 18 patients were analysed by the newer version marketed as Calprest.**

Results **No definite differences between pre- and post-endoscopy calprotectin were found, but it was considered preferable in the subsequent patients to analyse pre-endoscopy faeces. Upper-gastrointestinal disorders showed little difference in calprotectin levels, Barrett's oesophagus (median 6.8 mg/l), gastric ulcer (median 6.5 mg/l) or gastritis/duodenitis (median 5.2 mg/l), but these levels were all higher than the median calprotectin level of normal subjects (4.5 mg/l). The oesophageal and gastric carcinoma median was elevated significantly at 30 mg/l. Inflammatory bowel disease was also associated with marked elevation (Crohn's disease, 31.2 mg/l; ulcerative colitis, 116.2 mg/l). Colorectal polyps (median 3.7 mg/l) and adenoma (median 3.8 mg/l) showed no elevated levels in contrast to colorectal carcinoma (median 53.4 mg/l). The elevated calprotectin in inflammatory bowel disease and colorectal carcinoma combined gave a sensitivity of 81.8% and a specificity of 73.2%.**

Conclusions **Calprotectin levels are elevated in inflammation and cancer but are not helpful in differentiating between these disorders. In our series, calprotectin was not elevated in colonic polyps or adenomata. Calprotectin could be helpful as a screening method in a general gastroenterology population for inflammatory bowel disease and those with carcinoma, as well as assessing and monitoring disease activity in inflammatory bowel disease.** ***Eur J Gastroenterol Hepatol*** **14:841–845 © 2002 Lippincott Williams & Wilkins**

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Keywords: calprotectin, screening, oesophageal and gastric inflammation, inflammatory bowel disease, carcinoma

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Introduction

With increasing demands on endoscopic services, and a renewed emphasis on detecting precancerous lesions and early carcinoma, we have been seeking other methods of screening patients for intestinal disease. We have previously described technetium-labelled sucralfate for detecting and determining the extent of inflammatory bowel disease [1] as well as demonstrating its ability to detect peptic ulcers [2]. However, like ^{111}indium and ^{99m}technetium hexamethyl propylene amine oxime white-cell scanning for inflammatory bowel disease [3], it entails administering a radioactive material, which mitigates against a screening procedure. Neither procedure helps in detecting non-inflammatory bowel disease or cancer.

Faecal occult blood testing has been shown to be effective in screening for large colonic polyps and

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carcinoma [4]. However, it relies on the occurrence of intestinal bleeding and, therefore, underestimates the incidence of polyps, small tumours and inflammatory disease. To avoid false-positive results, it also requires that the patient being screened follows a restricted diet before faecal collection.

We were interested in assessing the efficacy of the calcium-binding protein calprotectin, which is found in plasma and certain body fluids but is six times more concentrated in the faeces. Calprotectin is present in mucous membrane squamous epithelium but not in the normal intestinal mucosa. It is present in neutrophil granulocytes and macrophages, in which it forms 60% of the cytosol protein [5]. The bacterial content of the gut lumen acts as a stimulus to exudation of these cells into the gut, and the amount increases with inflammation whether due to infection, inflammatory bowel disease, medication or neoplasia. It has been suggested that calprotectin might therefore be considered as a screening method for detecting intestinal disease.

Our aim was to assess the ability to demonstrate elevated faecal calprotectin levels in inflammation and cancer throughout the intestinal tract. During the trial, the method of analysis of faeces was modified, and comparability of results with the two methods therefore had to be achieved before analysing further patients with known inflammatory bowel disease or cancer. The study was approved by the hospital ethical research committee.

Methods

Unselected patients referred from primary or secondary care with gastrointestinal symptoms and/or signs attending a district general hospital for upper or lower endoscopy at the hospital's day services unit were invited to participate in the study. Those giving informed written consent were requested to bring a recent sample of faeces for assay of calprotectin. Patients having colonoscopy or flexible sigmoidoscopy had bowel preparation with laxative the day before endoscopy, so faeces were collected approximately 48 h before attendance at the unit. It has been shown previously that calprotectin is stable in faeces at room temperature for up to 8 days [5].

On arrival at the clinical biochemistry laboratory, the faecal samples were frozen and later thawed in batches of six, homogenized in extraction solution, then centrifuged; the resulting supernatant was collected and frozen again. Subsequently, batches of 18 supernatant samples were thawed, diluted with an equal volume of diluting liquid, and assayed in duplicate for calprotectin by the Nycotest PhiCal enzyme-linked immunosorbent assay (ELISA) (Nycomed, Oslo, Norway), which uses an affinity-purified rabbit polyclonal antibody against calprotectin coupled with a standard ELISA system.

Since instrumentation of the gut or bowel preparation might result in an inflammatory response, the initial 30 patients were also asked to provide a sample of faeces passed 7–10 days after endoscopy to see whether this produced any change in calprotectin levels.

Faecal samples from the following 116 patients, provided before endoscopy, were analysed. As only one case of carcinoma was found in this group, it was decided to collect further faecal samples from patients with known inflammatory bowel disease and carcinoma to assess the sensitivity using the manufacturer's upgraded kit. This provides an improved calprotectin assay requiring a smaller quantity of faeces and simplified faeces extraction procedure [6]. This necessitated reanalysis of some of the previous samples by the new method to assess comparability of results, after which a further 18 cases with known pathology were analysed by the new method, now marketed under the name of Calprest.

Calprotectin results for the different clinical groups were compared with the normal patients using the Mann–Whitney *U* test.

Results

The initial group of patients was studied to compare pre- and post-endoscopy levels of calprotectin. Seventeen of these 30 patients provided before and after faecal samples for analysis. The results did not reveal any consistent influence of endoscopy, although there was some variability probably attributable to biological and between-assay variation [5], but the possibility of some effect by the bowel preparation could not be excluded (Table 1). Subsequent patients were there-

Table 1 Faecal calprotectin concentrations in 17 patients pre- and post-endoscopy

Patient	Type of endoscopy	Faecal calprotectin (mg/l)	
		Pre-endoscopy	Post-endoscopy
1	Colonoscopy	3.2	4.1
2	Colonoscopy	37.0	50.8
3	Colonoscopy	25.0	9.2
4	Colonoscopy	5.0	3.6
5	Colonoscopy	16.9	28.4
6	Colonoscopy	19.6	22.3
7	Colonoscopy	4.7	5.2
8	Colonoscopy	34.0	61.6
9	Gastroscopy	76.4	23.6
10	Gastroscopy	13.7	18.6
11	Gastroscopy	13.0	9.4
12	Gastroscopy	9.9	7.6
13	Gastroscopy	116.8	110.8
14	Gastroscopy	4.6	2.8
15	Gastroscopy	15.8	12.1
16	Gastroscopy	3.5	6.4
17	Sigmoidoscopy	13.1	17.1

fore requested to provide faecal samples before endoscopy.

A further 116 patients were categorized according to their clinical and endoscopic diagnoses: 43 had upper-gastrointestinal lesions, seven had inflammatory bowel disease, seven had irritable bowel syndrome, 31 had colonic disorders (15 diverticular disease, 12 polyps, four adenomas), and 28 were endoscopically normal (Table 2). The 26 patients with upper-gastrointestinal inflammation due to gastritis and duodenitis had a median calprotectin level of 5.2 mg/l, which was not dissimilar to that seen in Barrett's oesophagus (6.8 mg/l) and gastric ulcer (6.5 mg/l). Although the level was higher than that for normal patients (4.5 mg/l), it was not statistically significantly different ($P = 0.115$). In colonic disorders, elevation of calprotectin was recorded in diverticular disease (median 11.1 mg/l; $P = 0.008$), but irritable bowel syndrome (median 6.1 mg/l; $P = 0.375$) did not reach statistical significance.

The final group of 18 patients consisted of seven with inflammatory bowel disease, two with colorectal adenoma, eight with colorectal carcinoma, and one with gastric carcinoma. Their calprotectin levels by the new method of analysis have been incorporated with the results of the 116 patients by using the accepted upper limit of normal by the old method of 10 mg/l and the new method of 50 mg/kg for the conversion [7,8]. Statistically significant calprotectin levels were observed in ulcerative colitis ($P < 0.0001$) and, to a lesser degree, in Crohn's disease ($P = 0.003$). Using the cut-off level of 10 mg/l (50 mg/kg new method) gave a sensitivity for detection of inflammatory bowel disease of 78.6%. Colorectal carcinoma also showed an elevated median calprotectin level of 53 mg/l ($P = 0.0005$), with a sensitivity of 87.5%. As inflammatory bowel disease and colorectal cancer were both present in the population of patients studied, it is not possible to quote meaningful specificity for calprotectin. If the two conditions are combined into one pathology category, then the sensitivity for detection of these patients was 81.8% with a specificity of 73.2%. The detailed results are shown in Table 2 and also depicted in Figure 1.

Table 2 Faecal calprotectin concentration (mg/l) in different disorders

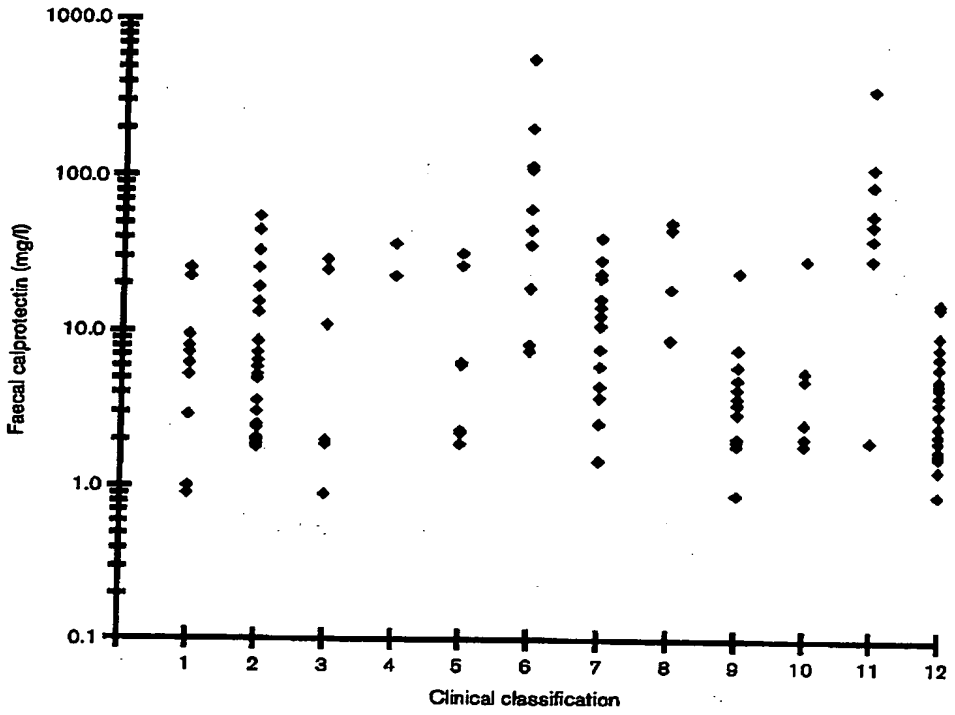
	Barrett's	Upper-GI inflammation	Gastric ulcers	Upper-GI tract cancer	IBS	Ulcerative colitis	Diverticular disease	Crohn's disease	Polyps	Colon adenoma	Colorectal cancer	Normal
Mean	8.9	12.0	11.7	30.0	11.0	116.2	13.5	31.2	5.4	7.8	94.1	5.1
SD	8.51	14.67	12.63	10.18	12.65	165.83	11.20	20.28	6.23	10.71	116.91	3.90
No. of subjects	10	26	6	2	7	10	15	4	12	6	8	28
Median	6.8	5.2	8.5	30.0	6.1	53.4	11.1	32.7	3.7	3.8	53.5	4.5
Range	0.8–25.7	1.8–54.9	0.9–29.4	22.8–37.2	1.9–32.0	7.8–555.2	1.6–40.4	9.0–50.2	0.9–24.2	1.9–29.4	2.0–370.4	0.9–15.5

Discussion

Calprotectin was initially described and assayed by Dale *et al.* [9], and a kit was subsequently developed and marketed by Nycomed. This allowed us to investigate the potential of faecal calprotectin as a screening technique for alimentary disease by analysing faeces provided by patients who had been referred for endoscopy.

Previous studies by Røseth *et al.* [5] included one oesophageal carcinoma and two gastric carcinomas and subsequently a mention of a further 18 patients with

Fig. 1



Logarithmic plot of faecal calprotectin results in different clinical conditions. Patient groups: Barrett's oesophagus, upper-gastrointestinal inflammation, gastric ulceration, cancer of the upper-gastrointestinal tract, irritable bowel syndrome, ulcerative colitis, diverticular disease, Crohn's disease, polyposis, colon adenoma, colorectal cancer and normal subjects.

gastric carcinoma [10]. The survey of patients by Campbell *et al.* [11] included coeliac disease and pancreatitis. In the present survey, other gastrointestinal disorders of the oesophagus, stomach and duodenum were assessed.

The level of calprotectin in patients with upper-gastrointestinal inflammation suggests that the response to ulceration is not producing significantly more calprotectin than a diffuse inflammation in the other conditions. Calprotectin was elevated significantly at 37.2 mg/l in the one case of squamous carcinoma oesophagus, but this was similar to two patients with benign stricture of the oesophagus (32.9 and 25.7 mg/l), not allowing for differentiation between a benign and malignant cause of dysphagia. However, as noted previously, active inflammatory bowel disease is a condition that produces the greatest rise in calprotectin [12,13]. In our series, the highest levels were recorded in one patient with total colitis (202.6 mg/l) and another with colitis before urgent colectomy (555.2 mg/l), suggesting that the level of calprotectin could be used to analyse the severity of the disease process.

The patients with colorectal carcinoma had elevated calprotectin levels (median 53.4 mg/l). As in previous reports [14–16], there was no significant difference according to colonic site. One patient with ascending carcinoma colon had a normal level. Since carcinoma cells do not synthesize calprotectin, this normal reading suggests that an exudative response had not been provoked. Nevertheless, the sensitivity and specificity of calprotectin in colorectal carcinoma were similar to those in previous reports [10,16,17]. The levels of calprotectin in patients who had colonic polyps or adenomas were within the normal range, presumably a reflection of the limited inflammatory response these polyps were producing within the colonic mucosa. Low calprotectin levels (median 9.1 mg/l) in patients with colonic polyps and adenomas were reported by Kronborg *et al.* [15], but in contrast our level in colonic carcinoma was much higher than they recorded. Tibble *et al.* [17] have shown in colorectal carcinoma a higher sensitivity for calprotectin than faecal occult blood testing but much lower sensitivity in colonic adenomas with a median calprotectin of 12 mg/l but only 4.5 mg/l in polyps.

Patients with irritable bowel syndrome had calprotectin levels similar to those with upper-gastrointestinal in-

flammation. This suggests altered mucosal function, allowing increased macrophages and granulocytes to pass into the lumen, and may be a reflection of the inflammatory and immunological changes described in this condition [18]. There were three endoscopically normal patients with unexplained mildly elevated calprotectin levels (11.4, 14.7 and 15.1 mg/l) and conversely no elevation in one patient with carcinoma colon. The main causes of elevated calprotectin were inflammatory bowel disease and carcinoma.

In view of the apparent relationship in inflammatory bowel disease between calprotectin level and the severity of illness, it should prove helpful in assessing and monitoring the degree of disease activity.

If calprotectin is used in an unselected district general hospital population as a screening method for alimentary disease, then it will pick up those with intestinal inflammation without specifying diagnosis but signalling the need for further investigation. It will also identify those with carcinoma of the upper or lower gastrointestinal tract. However, in our experience, it will not alert the investigator to the presence of colonic polyps or adenoma.

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Diagnostic Precision of Fecal Calprotectin for Inflammatory Bowel Disease and Colorectal Malignancy

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OBJECTIVES: Fecal calprotectin (FC) is a relatively new marker of intraluminal intestinal inflammation. Using meta-analytical techniques, the study aimed to evaluate the diagnostic precision of FC for inflammatory bowel disease (IBD) and colorectal cancer (CRC) in adults and children.

METHODS: Quantitative meta-analysis was performed on prospective studies, comparing FC levels against the histological diagnosis. Sensitivity, specificity, and diagnostic odds ratio (DOR) were calculated for each study. Summary receiver-operating characteristic (sROC) curves and subgroup analysis were undertaken. Study quality and heterogeneity were evaluated.

RESULTS: Thirty studies of 5,983 patients were included. FC levels in patients with IBD were higher by 219.2 micrograms per gram (μg/g) compared with normal patients ($P < 0.001$). sROC curve analysis showed a sensitivity of 0.95 (95% CI 0.93–0.97), specificity of 0.91 (95% CI 0.86–0.91), and an area under the curve (AUC) of 0.95 for the diagnosis of IBD. Patients with colorectal neoplasia had nonsignificantly higher FC levels by 132.2 μg/g compared with noncancer controls ($P = 0.18$). Sensitivity and specificity of FC for the diagnosis of CRC were 0.36 and 0.71, respectively, with an AUC of 0.66. The diagnostic precision of FC for IBD was higher in children than adults with better accuracy at a cutoff level of 100 μg/g *versus* 50 μg/g. Sensitivity analysis and metaregression analysis did not significantly alter the results.

CONCLUSIONS: FC cannot be recommended as a screening test for CRC in the general population. FC appeared to offer a good diagnostic precision in distinguishing IBD from non-IBD diagnoses, with higher precision at a cutoff of 100 μg/g.

(Am J Gastroenterol 2007;102:803–813)

INTRODUCTION

Fecal calprotectin (FC) has recently emerged as a candidate biomarker of intestinal inflammation with potential clinical application as a diagnostic adjunct in inflammatory bowel disease (IBD) and other pathologies of the gastrointestinal tract (1). First described by Fagerhol *et al.* in 1980 (2), it has also been known as L1 protein (3), MRP-8/14 (4), calgranulin (5), and cystic fibrosis antigen (6). It is a 36 kiloDalton calcium-binding protein with antimicrobial and antiproliferative properties, which comprises 60% of cytosolic protein in neutrophil granulocytes (7). Calprotectin is excreted in feces and can be measured with a commercially available ELISA immunoassay (8) at a cost of US$ 40–60 per assay (9, 10). The protein is stable in feces for up to seven days at room temperature and the test can be performed on 1–2 g of stool, enabling sample collection at home and delivery to the laboratory by post if necessary (1, 11).

FC levels have been found to be significantly elevated in patients with inflammatory and neoplastic conditions, including Crohn's disease (CD), ulcerative colitis (UC), and colorectal cancer (CRC) (11, 12). Levels of FC have been found to correlate well with radio-labeled white cell scanning, used for assessing active intestinal inflammation in CD (13), as well as with histological and endoscopic assessment of disease activity in UC (14). A number of studies have also assessed the sensitivity and specificity of FC in the diagnosis of IBD and colorectal neoplasia. Pediatric gastroenterologists may be particularly interested in the use of FC, as diagnostic tests distinguishing organic from functional disorders may help to minimize the use of invasive procedures in children (9).

Using meta-analytical techniques, the present study aimed: (a) to evaluate the diagnostic precision of FC in the adult and pediatric population with particular reference to IBD and CRC, (b) to examine possible differences in the diagnostic precision of FC in adults *versus* pediatric patients, and

(c) to identify possible cutoff levels at which the FC assay offers the optimum diagnostic precision.

METHODS

Search Strategy and Selection Criteria

A computerized search of Ovid, EMBASE, the Cochrane Database, and MEDLINE was conducted (using PubMed as the search engine) in order to identify studies on the use of FC in the diagnosis of gastroenterological conditions. The following search terms were used: "calprotectin and IBD," "calprotectin and Crohn's disease," "calprotectin and ulcerative colitis," "calprotectin and cancer," "calprotectin and intestinal inflammation," "IBD and leukocyte L1 complex," and "calprotectin and feces." Databases were searched up until March 14th 2006, no lower date limit was applied, and no restrictions were made on language. Further articles were identified by use of the "related articles" function. References of all papers were retrieved in full, and review articles on FC were evaluated for the inclusion into the study by title and abstract examination.

Eligibility Criteria and Data Extraction

Human studies that compared FC with histological diagnosis of Crohn's disease (CD), ulcerative colitis (UC), and CRC, both in the adult and pediatric population, were included. Studies that compared FC with radio-labeled white cell scanning and clinical indices of disease activity for CD and UC were also included. Studies that did not feature a control group (healthy patients or those with irritable bowel syndrome) were excluded, as were those on neonates, studies measuring FC following drug, dietary, or surgical interventions, tissue studies, and animal studies. Studies for which there was a possibility of overlap of patient cohorts with other included studies and those evaluating technical aspects of the calprotectin assay were also excluded. Data were obtained on author, publication date, study design, patient characteristics, diagnostic groups compared, type of assay used, and sensitivity and specificity at various cutoff values. Data were extracted independently by two authors (AvR and LK); in cases of discordance, a consensus was reached through discussion with the senior author (PPT). Study quality was assessed with the QUADAS (quality assessment for studies of diagnostic accuracy) tool (15), in which a score of one was given if a criterion was fulfilled, zero if it was unclear, and minus one if it was not achieved.

End Points and Definitions

The primary end points were the pooled weighted mean difference (WMD), sensitivity, specificity, diagnostic odds ratio (DOR), and area under the summary receiver-operating characteristic curve (sROC) of FC in patient populations with Crohn's disease, ulcerative colitis, colorectal neoplasms, irritable bowel syndrome, and healthy controls.

Statistical Analysis

In 2000, a new assay for FC became widely available, which was five times as sensitive as the original assay and measured FC in micrograms per gram rather than milligrams per liter (8). A number of authors have asserted that results obtained with the old assay method may be directly compared with results obtained by the new method through simply multiplying the former by a factor of five (8, 16, 17). In order to verify this, the manufacturers (Calpro AS, Oslo, Norway) were contacted directly, who confirmed that results could be translated in the manner described above. To allow inclusion of all studies in the common analysis, we therefore applied a factor of five to FC values obtained from studies that used the original assay. Meta-analysis was performed in accordance with previously reported guidelines for meta-analyses of diagnostic tests (18–20). Pooled estimates for the difference in calprotectin values among different diagnoses were obtained by calculating the WMD with 95% confidence intervals, assuming a random-effects model (21).

In the diagnostic precision calculations, sensitivity and specificity of FC in distinguishing between patients with IBD or colorectal neoplasia and patients in the control group were extracted or calculated by the use of 2×2 contingency tables of correct diagnosis and FC level above the specified cutoff value. Pooled estimates were calculated using a random-effects model. sROC analysis was performed to assess the interaction between sensitivity and specificity (22). DOR, Q-statistic, and area under the sROC curve were used to analyze the diagnostic precision of FC in distinguishing the diagnosis of interest from the control group. DOR was calculated from data for sensitivity and specificity, and was defined as: (frequency of true positives $\div$ frequency of false positives) $\div$ (1 $-$ frequency of true positives $\div$ 1 $-$ frequency of false positives). The higher the DOR, the greater the diagnostic precision of FC in distinguishing cases from controls.

The Q-statistic is a χ^2 test that measures heterogeneity among studies, and was used to calculate the applicability of the sROC regression over the dataset. The area under the sROC curve measured test precision of FC; a value of 0.5 indicated a test that was equally likely to diagnose a positive result as either positive or negative. A test which gave the correct diagnosis in 100% of cases had an AUC of 1.0. Most tests achieve a value of between 0.5 and 1.0, with better diagnostic precision correlating with an AUC closer to 1.0. Subgroup analysis and weighted meta-regression were used to assess the diagnostic precision of FC between adults and children and compare two cutoff levels for test positivity at 50 and 100 μg/g.

Statistical analysis was performed with the statistical software packages Review Manager, version 4.2 (The Cochrane Collaboration®, Software Update, Oxford, UK), STATA version 9.1SE (StataCorp LP, College Station, Texas), and MetaDiSc version 1.1.1.

RESULTS

A total of 233 reports were identified by the literature search of which 45 were selected for full review (Fig. 1). Following exclusion of 15 studies, 30 were included in the final analysis (11–14, 16, 23–47), the characteristics of which are summarized in Table 1. A total of 5,983 patients underwent FC testing. Of these, 663 patients had a diagnosis of CD, 361 had UC, 186 were labeled as having IBD, 297 had CRC, 697 had IBS and 3,393 were controls.

Seven studies measured FC solely in the pediatric population (24, 29, 30, 34, 40, 46, 47), one measured it in both children and adults (38), and 22 studies were performed on adults only (11–14, 16, 23, 25–28, 31–33, 35–37, 39, 41–45). Two studies assessed the diagnostic precision of FC in predicting relapse in CD and UC (28, 42), three examined diagnostic precision in the assessment of disease activity in IBD (26, 27, 47), and four did not contain extractable data for diagnostic precision (11, 13, 14, 40).

Table 2 shows the pooled difference in FC levels among various diagnostic groups, using WMD as the effect measure. FC levels were higher in patients with IBD when compared with healthy controls by 219.23 μg/g (95% CI 174.49–263.97, P <0.001). There was no significant difference in FC values between patients with CRC and those without CRC (WMD 132.19 μg/g, 95% CI −59.18–323.56, P = 0.18). There was no significant difference in FC levels when comparing patients with IBS with healthy controls (WMD −4.01, 95% CI −21.63–13.61, P = 0.66). In patients with CD, FC levels were significantly higher than in patients with IBS by 303.67 μg/g (95% CI 167.50–439.83, P < 0.001). Significant interstudy heterogeneity was evident for all compared diagnostic categories.

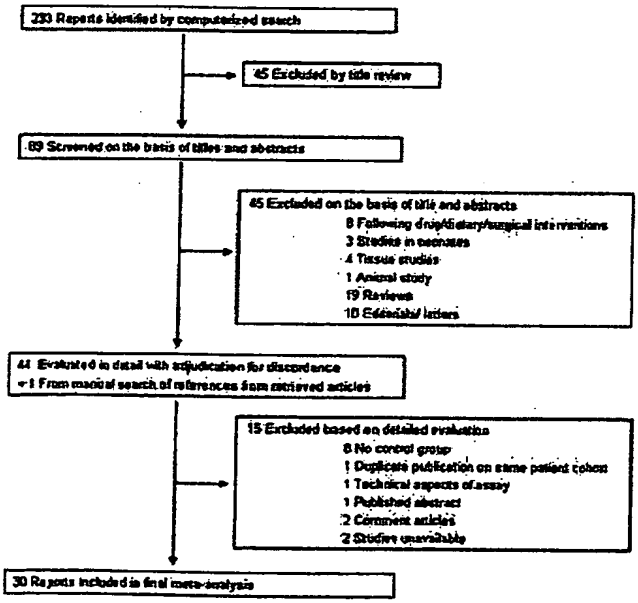

Figure 1. Study selection.

Results of the pooled sensitivity, specificity, DOR, and AUC of FC in distinguishing between IBD and colorectal neoplasia, at cutoff values of 50 μg/g and 100 μg/g, for adult and pediatric subgroups are shown in Table 3. The overall weighted AUC of FC in distinguishing patients with IBD from patients who did not have IBD for a total of 1,267 adults and children was 0.95 (SE 0.02, DOR 75.57, 95% CI 29.80–191.61), with significant interstudy heterogeneity (Q-value 61.75, P < 0.001). Forest plots of the pooled sensitivity and specificity are shown in Figure 2. When a 100 μg/g cutoff was assessed, the AUC was increased to 0.98 (SE 0.03, DOR 264.96, 95% CI 39.78–1,765.00), with no significant heterogeneity (Q-value 6.48, P = 0.091). In the pediatric population, a cutoff value of 50 μg/g for distinguishing patients with IBD from those without IBD also gave an improved AUC of 0.96 (SE 0.02, DOR 94.79, 95% CI 30.14–298.09), with no significant heterogeneity (Q-value 0.89, P = 0.640). When the cutoff level was set at 100 μg/g, the AUC increased to 0.99 (SE 0.01, DOR 496.8, 95% CI 36.20–6825.60), with no significant heterogeneity (Q-value 5.17, P = 0.075).

The overall AUC in distinguishing adult and pediatric patients with CD from patients with IBS and healthy control patients at a cutoff value of 50 μg/g was 0.97 (SE 0.01, DOR 129.00, 95% CI 44.12–377.18), with no significant heterogeneity (Q-value 11.41, P = 0.07). The pooled DOR for distinguishing patients with UC from those with IBS and healthy controls was 15.29 (95% CI 7.52–31.08), with no significant heterogeneity (Q-value 0.60, P =0.440). The pooled analysis of diagnostic precision of FC in distinguishing colorectal neoplasms from nonneoplastic disorders (including inflammatory conditions) demonstrated an AUC of 0.66 (SE 0.05, DOR 2.77, 95% CI 1.38–5.55). When patients with inflammatory conditions were excluded from the "no neoplasia" group, FC appeared to have better diagnostic precision for detecting CRC (AUC 0.88, SE 0.07, DOR 17.01, 95% CI 3.18–91.83) than for detecting adenomas (AUC 0.76, SE 0.12, DOR 5.67, 95% CI 0.55–58.65) at a cutoff of 50 μg/g. All analyses for colorectal neoplasia demonstrated significant interstudy heterogeneity. sROC curves comparing the diagnostic precision of FC in IBD and colorectal neoplasia are shown in Figure 3.

Publication bias was assessed by means of a funnel plot of studies included in the metaanalysis of WMD of FC levels in patients with IBD compared with healthy controls (Fig. 4). Individual study estimates were evenly clustered around the pooled estimate of 219.23 μg/g, suggesting a lack of publication bias. The large number of studies lying outside the 95% confidence limits reflects the relatively high degree of observed interstudy heterogeneity.

Sensitivity analysis was performed to assess the impact of high-quality studies (QUADAS score above 11) and large studies (more than 100 patients) on sensitivity, specificity, DOR, and interstudy heterogeneity (Table 4). When studies of lower quality were excluded, the sensitivity of FC in

Table 1. Study Characteristics

Ref	Author (year)	Pop	Number of Patients: CD	UC	IBD	CRC	IBS	C	Inclusion Criteria	Design	Assay	Gold Standard	Diagnostic: Cutoff*	Precision	Quality
11	Roseth (1992)	A	21	17	—	—	—	33	4,5,6	P	OM	N/A	N/A	—	11
12	Roseth (1993)	A	—	—	—	53	—	64	4,14,17	P	OM	Histo	50	√	10
13	Roseth (1999)	A	19	10	—	—	—	9	4,6	P	OM	N/A	N/A	—	11
14	Roseth (1997)	A	—	62	—	—	—	125	4,5	P	OM	N/A	N/A	—	13
16	Summerton (2002)	A	4	10	—	8	7	28	16	P	OM + NM	Histo	50	√	12
23	Kristinsson (2001)	A	—	—	—	5	—	114	17	P	OM	Histo	50	√	13
24	Canani (2006)	C	17	10	—	—	—	18	1	R	NM	Histo	100	√	13
25	Silberer (2005)	A	—	—	39	—	40	40	2,3,4	P	NM	Histo	18.6	√	13
26	Langhorst (2005)	A	—	31	—	—	—	—	5	P	OM	CAI	30	†	12
27	Gaya (2005)	A	35	—	—	—	—	—	6,7	P	NM	WCS	100	†	10
28	Costa (2005)	A	38	41	—	—	—	—	5,6	P	NM	CDAI/UCAI	150	‡	10
29	Fagerberg (2005)	C	—	—	22	—	—	—	1	P	NM	Histo	50	√	13
30	Bremner (2005)	C	—	—	43	—	—	7	4,5,6,8,9	R	NM	Histo	50	√	10
31	Wassell (2004)	A	25	—	—	—	25	27	3,4,6	P	NM	Histo	90	√	11
32	Hoff (2004)	A	—	—	—	16	—	1518	10	P	NM	Histo	50	√	10
33	Dolwani (2004)	A	25	—	—	—	24	26	3,4,6	P	NM	Histo	60	√	13
34	Canani (2004)	C	68	45	—	—	—	76	4,11	P	NM	Histo	100	√	11
35	Thjodleifsson (2003)	A	49	—	—	—	—	163	6,12,13	P	OM	Histo	50	√	11
36	Limburg (2003)	A	—	—	—	97	—	148	14	P	NM	Histo	50	√	13
37	Costa (2003)	A	49	82	—	26	48	34	4,11	P	NM	Histo	50	√	12
38	Carrocio (2003a)	A	10	—	—	3	40	10	4,6,15	P	NM	Histo	50	√	12
38	Carrocio (2003b)	C	8	—	—	—	15	10	4,6,15	P	NM	Histo	50	√	12
39	Tibble (2002)	A	84	—	—	7	339	—	11	P	OM	Histo	50	√	13
40	Olafsdottir (2002)	C	—	—	17	—	—	24	1,2,4,11	R	NM	N/A	N/A	—	11
41	Tibble (2001)	A	—	—	25	62	—	96	4,14,17	P	OM	Histo	50	√	12
42	Tibble (2000)	A	43	37	—	—	—	—	20	P	OM	CDAI/HBI	250	‡	12
43	Tibble (2000a)	A	31	—	—	2	159	—	1	P	OM	Histo	150	√	12
43	Tibble (2000b)	A	116	—	—	—	—	56	4,6	P	OM	Histo	50	√	12
44	Limburg (2000)	A	—	—	29	—	—	81	15	P	NM	Histo	100	√	11
45	Kronborg (2000)	A	—	—	—	23	—	488	21	P	OM	Histo	50	√	11
46	Bunn (2001)	C	21	16	—	—	—	31	4,5,6	P	OM	Histo	50	√	12
47	Bunn (2001)	C	—	—	36	—	—	—	14,19	P	OM	WCS/ Histo	31.5	†	12

Pop = study population; C = children; A = adults; CD = Crohn's disease; UC = ulcerative colitis; IBD = inflammatory bowel disease; CRC = colorectal cancer; IBS = irritable bowel syndrome; C = controls; P = prospective; R = retrospective; NM = new assay method; OM = old assay method; Histo = histology; CAI = colitis activity index; WCS = white cell scan; CDAI = Crohn's disease activity index; UCAI = ulcerative colitis activity index; HBI = Harvey Bradshaw index; N/A = not applicable; * = given in micrograms per gram; √ = primary diagnosis; † = diagnosis of disease activity; ‡ = diagnosis of relapse.
Inclusion criteria:
1= suspected inflammatory bowel disease; 2 = known chronic inflammatory bowel disease; 3 = irritable bowel syndrome; 4 = asymptomatic patients; 5 = ulcerative colitis; 6 = Crohn's disease; 7 = active inflammatory bowel disease; 8 = constipation; 9 = other gastrointestinal diagnoses; 10 = colorectal cancer screening; 11 = consecutive patients referred for assessment of gastrointestinal symptoms; 12 = first-degree relatives of patients with Crohn's disease; 13 = spouses of patients with Crohn's disease; 14 = referred for colonoscopy; 15 = chronic diarrhea; 16 = referred for upper or lower gastrointestinal endoscopy; 17 = known colorectal malignancy; 18 = first-degree relatives of patients with colorectal cancer; 19 = patients undergoing mdio-labelled white-cell scanning; 20 = inflammatory bowel disease in clinical remission; 21 = high-risk patients in colorectal cancer screening.

diagnosing IBD in adults at a cutoff level of 50 μg/g increased from 0.71 to 0.90 (95% CI 0.86–0.93). Specificity was unaffected at 0.80, the DOR increased from 55.07 to 65.70 (95% CI 15.78–274.29), and the AUC remained similar, at 0.95 (SE 0.03). Excluding lower quality studies also increased the sensitivity of FC in diagnosing colorectal neoplasia from 0.36 to 0.62 (95% CI 0.54–0.68); however, specificity was reduced from 0.74 to 0.66 (95% CI 0.63–0.70). The DOR increased from 2.57 to 4.46 (95% CI 0.78–25.37) and the AUC increased from 0.65 to 0.73 (SE 0.12). When only large studies were included, the sensitivity of FC in diagnosing IBD in adults at a cutoff level of 50 μg/g increased from 0.71 to 0.90 (95% CI 0.86–0.93). Specificity was similar at 0.82 (95% CI 0.78–0.85), the DOR increased from 55.07 to 103.48 (95% CI 19.08–561.26), and the AUC was similar at 0.96 (SE 0.02). Although there was a trend toward reduction of interstudy heterogeneity in the sensitivity analysis, heterogeneity could not be eliminated by the exclusion of low-quality and small studies. The effect of interstudy heterogeneity was assessed by weighted meta-regression analysis of DOR against significant study variables in the analysis distinguishing patients with IBD from those without IBD: the cutoff value used for FC (100 μg/g or 50 μg/g, 1,691 patients), study population (children or adults), quality (QUADAS $\geq$ 12 *vs.* others), and the type of assay used (new or old assay method) did not have a statistically significant effect on the DOR.

Table 2. Pooled Fecal Calprotectin Levels When Comparing Various Diagnostic Groups

Outcome	No. of Patients	No. of Studies	WMD (μg/g)	95% CI	*P* Value	HG (χ^2)	HG (*P* Value)
IBD *vs.* normal	1,516	23	219.23	174.49–263.97	<0.001	532.98	<0.001
CD *vs.* normal	803	12	170.14	162.06–178.21	<0.001	138.17	<0.001
UC *vs.* normal	479	7	186.19	45.59–326.80	0.009	196.33	<0.001
IBS *vs.* normal	294	6	−4.01	−21.63–13.61	0.660	61.86	<0.001
CRC *vs.* no CRC	2,661	7	132.19	−59.18–323.56	0.180	1193.33	<0.001
CD *vs.* UC	453	8	50.73	3.33–98.14	0.040	40.76	<0.001
CD *vs.* IBS	829	8	303.67	167.50–439.83	<0.001	240.91	<0.001

IBD = inflammatory bowel disease; CD = Crohn's disease; UC = ulcerative colitis; IBS = irritable bowel syndrome; CRC = colorectal cancer; No = number; WMD = weighted mean difference; μg/g = micrograms per gram; CI = confidence interval; HG = heterogeneity.

DISCUSSION

The present study demonstrated that levels of FC in patients with IBD are significantly different from the normal patient population. No significant difference in FC levels could be detected between patients with CRC and those without CRC, or between FC levels in patients with IBS and healthy patients. Although FC levels in patients with CD were higher than in those with UC by 55.79 μg/g, this finding is of limited clinical significance because the range of FC values in both CD and UC is large, and the test is not useful for differentiating between the two conditions (39).

With regard to the diagnostic precision of FC, the present study shows that the test can potentially discriminate between patients with IBD and those without IBD, and is equally good in children and adults. The analysis also demonstrates that at a cutoff of 100 μg/g (AUC 0.98, DOR 264.96), the diagnostic precision of FC for IBD appears to be better than at a cutoff of 50 μg/g (AUC 0.95, DOR 75.57). FC also appears to have good diagnostic precision in differentiating patients with CD from those with IBS (AUC 0.97), again with a tendency towards higher precision at a cutoff of 100 μg/g. FC was poor at separating colorectal neoplasia from nonneoplastic conditions including inflammation (AUC 0.66). When inflammatory conditions were excluded from the control group, FC was better at diagnosing cancers (AUC 0.88) than adenomas (AUC 0.76).

Two of the included studies assessed the diagnostic accuracy of FC in predicting clinical relapse in CD and UC. Tibble *et al.* found that, at a cutoff rate of 50 mg/L ($\equiv$ 250 μg/g), FC had a sensitivity of 90% and a specificity of 83% in predicting clinical relapse in 80 IBD patients who were in remission over a 12-month period (42). Costa *et al.* reported similar results for FC in the prediction of relapse in 41 UC patients over 12 months (89% sensitivity, 82% specificity), but found FC levels to be less precise in predicting relapse in 38 CD patients (87% sensitivity, 43% specificity) over the same time period (28). The results are not directly comparable, as a lower cutoff level of 150 μg/g was used in the latter study. Nonetheless, FC appears to have a good diagnostic precision in predicting relapse in IBD, possibly more so in UC than in CD.

Two included studies correlated FC levels with fecal excretion of radio-labeled white cells in CD (13, 43). Both found the two tests to be strongly correlated ($r = 0.87$, $P < 0.001$ and $r = 0.80$, $P < 0.001$). A further study, not included in the meta-analysis, found that of 45 IBD patients with FC levels below 50 μg/g, 44 had endoscopically normal colonic mucosa, and 7 showed only mild inflammation on histology (17). Hence, FC appears to have potential as a noninvasive marker of disease activity in IBD.

Two studies attempted correlation of FC levels with clinical disease activity scores. Costa *et al.* found that FC levels above 50 μg/g were better correlated with the colitis activity index ($r = 0.60$, $P < 0.001$) than with the Crohn's disease activity index (CDAI) ($r = 0.44$, $P < 0.01$) (37). Gaya *et al.* did not find a correlation between FC levels and CDAI ($r = 0.33$, $P = 0.06$) (27). The exact strength of any correlation of FC levels with clinical disease activity indices is therefore not well established at present, and further studies are required to resolve this issue.

The precision of FC for the diagnosis of IBD appears to be superior to serological markers such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), anti-*Saccharomyces cerevisiae* antibody (ASCA), perinuclear antineutrophil cytoplasmic antibody (ANCA), and anti-*Escherichia coli* outer membrane porin C antibody (anti-OmpC). Four studies in the meta-analysis listed comparative values for CRP and ESR (24, 25, 43, 48). The sensitivity of CRP ranged from 35% to 40%, and specificity was 78–100%. For ESR, sensitivity ranged from 18% to 52%, and specificity was 78–100%. A recent meta-analysis found the combination of a positive ASCA test and a negative ANCA test to be 55% sensitive and 93% specific for diagnosing CD (AUC 0.85), whereas a positive ANCA test alone was found to be 55% sensitive and 89% specific for diagnosing UC (AUC 0.82) (49). Anti-OmpC has been reported to have a sensitivity of 11–55% for CD (50–53) and up to 11% for UC (50, 52). Data on specificity are limited, but this may be as high as 95% for both CD and UC (50).

To the authors' knowledge, this is the first study that employs meta-analytical techniques to quantify the diagnostic precision of FC for gastrointestinal disorders, although a number of reviews have been published on the subject (1, 9, 10, 54–56). The results of this meta-analysis must be interpreted with a number of limitations in mind. The pooling of diagnostic accuracy data inevitably introduces sources of bias (57), which were reflected in the significant amount of

Table 3. Diagnostic Precision of Fecal Calprotectin in Distinguishing Between Different Clinical Conditions at Various Cutoff Values

Pooled Analysis	Patients	Studies	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)	HG $\chi 2$ (HG *P* Value)	AUC (SE)	Reference
IBD *vs.* no IBD								
Adults & children (50 μg/g)	1,267	9	0.89 (0.86–0.91)	0.81 (0.78–0.84)	72.31 (19.55–267.49)	61.75 (<0.001)	0.95 (0.02)	16, 29, 30, 35, 37, 38, 41, 43, 46
Adults & children (100 μg/g)	328	4	0.98 (0.93–0.99)	0.91 (0.86–0.95)	264.96 (39.78–1,765.00)	6.48 (0.091)	0.98 (0.03)	24, 34, 36, 38
Adults (50 μg/g)	1,030	6	0.71 (0.67–0.75)	0.80 (0.77–0.83)	55.07 (10.20–297.38)	49.69 (<0.001)	0.94 (0.04)	16, 35, 37, 38, 41, 42
Children (50 μg/g)	201	3	0.83 (0.73–0.90)	0.85 (0.77–0.91)	77.40 (22.16–270.33)	0.89 (0.640)	0.96 (0.02)	30, 38, 46
Children (100 μg/g)	231	3	0.98 (0.94–1.00)	0.97 (0.92–0.99)	496.8 (36.20–6,825.60)	5.17 (0.075)	0.99 (0.01)	24, 34, 38
Crohn's disease *vs.* normal controls & IBS								
Adults & children (50 μg/g)	733	5	0.95 (0.92–0.97)	0.84 (0.80–0.87)	129.00 (44.12–377.18)	11.41 (0.070)	0.97 (0.01)	30, 35, 37, 38, 43
Adults (50 μg/g)	614	4	0.95 (0.91–0.97)	0.85 (0.81–0.89)	141.41 (33.79–591.78)	10.98 (0.027)	0.97 (0.02)	35, 37, 38, 43
Children (50 μg/g)	119	2	0.97 (0.86–1.00)	0.79 (0.69–0.87)	105.40 (18.20–610.80)	0.48 (0.487)	—	30, 38
Children (100 μg/g)	155	2	1.00 (0.93–1.00)	0.98 (0.93–1.00)	1165.30 (73.00–1,8602.00)	1.45 (0.229)	—	38, 34
Ulcerative colitis *vs.* normal controls & IBS								
Adults & children (50 μg/g)	235	2	0.78 (0.69–0.86)	0.78 (0.70–0.84)	15.29 (7.52–31.08)	0.60 (0.440)	—	30, 37
Colorectal neoplasia								
CRC & adenoma *vs.* no neoplasia, including inflammation (50 μg/g)	4,112	7	0.36 (0.34–0.39)	0.71 (0.70–0.73)	2.77 (1.38–5.55)	65.35 (<0.001)	0.66 (0.05)	32, 36, 37, 38, 41, 43, 45
Adenoma *vs.* no neoplasia, excluding inflammation (50 μg/g)	547	3	0.52 (0.42–0.61)	0.77 (0.73–0.81)	5.67 (0.55–58.65)	15.80 (<0.001)	0.76 (0.12)	12, 23, 43
Adenoma *vs.* no neoplasia, excluding inflammation (100 μg/g)	466	3	0.27 (0.20–0.35)	0.91 (0.88–0.94)	4.61 (0.56–37.94)	13.74 (0.001)	0.74 (0.14)	12, 23, 44
CRC *vs.* no neoplasia, excluding inflammation (50 μg/g)	2,025	4	0.87 (0.77–0.94)	0.76 (0.74–0.78)	17.01 (3.18–91.83)	12.25 (0.007)	0.88 (0.07)	12, 23, 32, 43

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; CRC = colorectal cancer; CI = confidence interval; DOR = diagnostic odds ratio; HG = heterogeneity; AUC = area under the curve; SE = standard error.

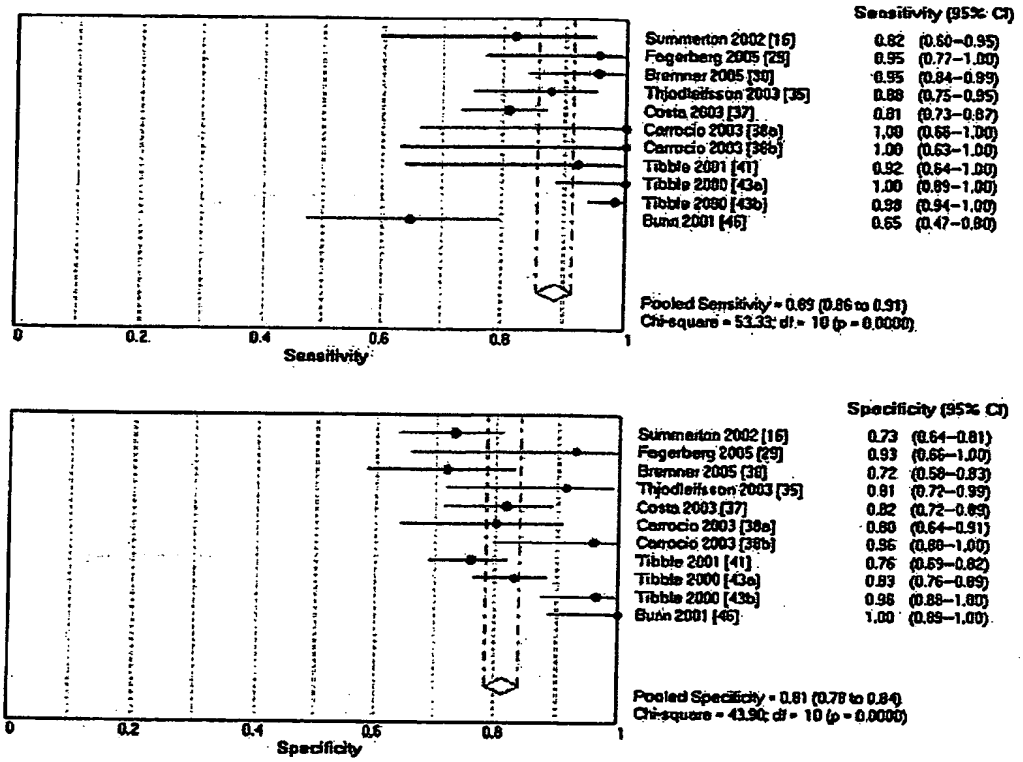

Figure 2. Forest plots of pooled sensitivity and specificity of fecal calprotectin in distinguishing inflammatory bowel disease (IBD) from non-IBD diagnoses at a cutoff level of 50 μg/g. CI = confidence intervals; df = degrees of freedom.

statistical heterogeneity observed in parts of the analysis. Foremost amongst these was spectrum bias, *i.e.*, the phenomenon that the sensitivity and specificity of a diagnostic test may change depending on the severity of disease in the tested population (58). Of the nine studies (16, 29, 30, 35, 37, 38, 41, 43, 46) included in the meta-analysis of diagnostic precision of FC in differentiating IBD from non-IBD diagnoses, only five (30, 35, 37, 38, 46) described disease severity in the IBD population by measuring CDAI or CAI, and all five studies differed in terms of the proportions of mild-to-severe

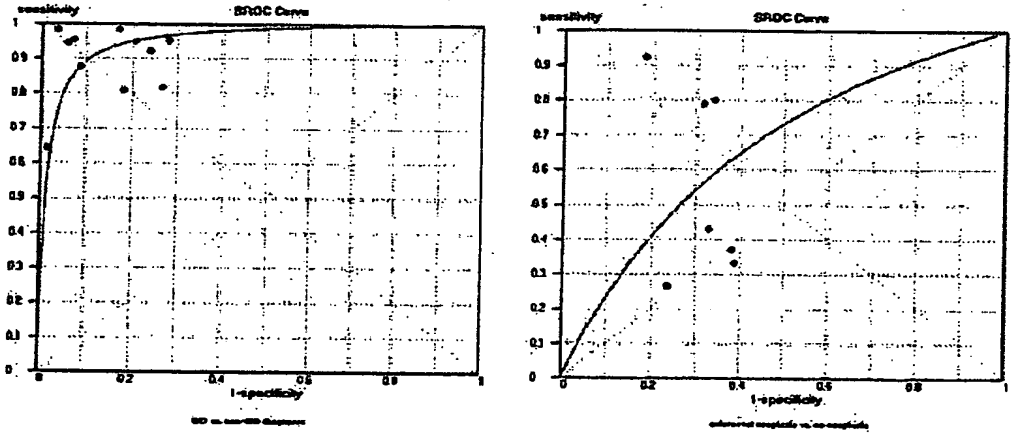

Figure 3. Summary receiver-operating characteristic (sROC) curves for fecal calprotectin in the diagnosis of inflammatory bowel disease (IBD) and colorectal neoplasia at a cutoff level of 50 μg/g.

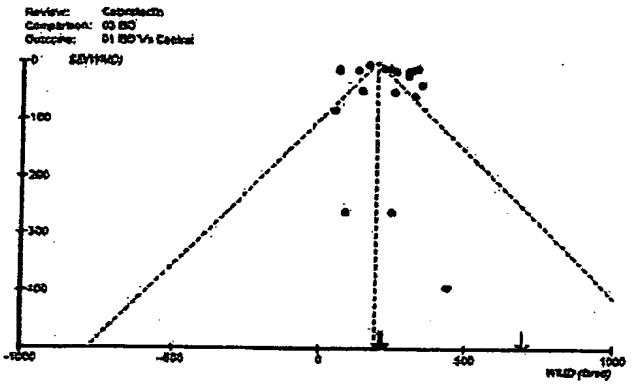

Figure 4. Funnel plot of the weighted mean difference (WMD) of fecal calprotectin values when comparing patients with inflammatory bowel disease (IBD) with healthy controls. This is a scatter plot of the weighted mean difference estimated in individual studies plotted on the horizontal axis, against the standard error of the estimate shown on the vertical axis (SE [WMD]).

disease in the IBD population being studied. However, none of these studies reported diagnostic precision results stratified by disease activity, or by pretest probability of IBD based on clinical assessment. The results of the present meta-analysis appear to suggest that FC has a good diagnostic precision for separating IBD from non-IBD diagnoses overall. Whilst this finding is likely to hold true in patients with severe IBD, it may not necessarily translate to a clinical setting where the patient has a low pretest probability of IBD, *i.e.*, where a clinician is attempting to differentiate patients with functional abdominal pain syndromes or IBS from IBD patients with mild, "functional-like" symptoms. The study by Tibble *et al.* (43) partially addresses this question by including only patients referred to a hospital gastroenterological outpatient practice for differentiation between IBD and IBS. At a cutoff of 30 mg/L (≡150 μg/g), the authors report a sensitivity of 100% and specificity of 97% for differentiating between CD and IBS. However, disease severity in the CD cohort is not described accurately, and the results may therefore still be subject to spectrum bias.

Anatomical disease distribution in both CD and UC may also affect the diagnostic precision of FC, and whilst the five studies mentioned above described disease distribution in the IBD cohort, none presented diagnostic precision results stratified by disease distribution.

Selection bias is likely to have been a further source of heterogeneity, as a number of different diagnoses were grouped together in some parts of the analysis, *e.g.*, the "no IBD" group contained patients with diverticular disease, IBS, food intolerance, and healthy patients. The original studies also varied in terms of inclusion and exclusion criteria as well as study design; factors that are known to have influence on estimates of diagnostic precision (59). Nevertheless, the high DORs demonstrated in most parts of the analysis indicate a strong trend and support the argument that FC may be a very useful clinical test for IBD.

Table 4. Sensitivity Analysis

Pooled Analysis	Patients	Studies	Sensitivity (95% CI)	Specificity (95% CI)	DOR (95% CI)	HG χ^2 (HG P Value)	AUC (SE)	Reference
High-quality studies—IBD *vs.* no IBD								
Adults & children (50 μg/g)	1,095	7	0.88 (0.85–0.91)	0.82 (0.79–0.85)	86.28 (26.37–282.33)	27.47 (0.001)	0.96 (0.02)	16, 29, 37, 38, 41, 43, 46
Adults (50 μg/g)	958	5	0.90 (0.86–0.93)	0.80 (0.77–0.83)	65.79 (15.78–274.29)	22.94 (<0.001)	0.95 (0.03)	16, 37, 38, 41, 43
High-quality studies—Crohn's disease *vs.* normal controls & IBS								
Adults & children (50 μg/g)	575	3	0.97 (0.93–0.99)	0.85 (0.81–0.89)	188.13 (35.45–998.35)	11.20 (0.024)	0.98 (0.02)	37, 38, 43
Adults (50 μg/g)	542	3	0.97 (0.93–0.99)	0.85 (0.80–0.88)	179.57 (25.10–1,284.80)	10.88 (0.012)	0.98 (0.02)	37, 38, 43
Large studies—IBD *vs.* no IBD								
Adults (50 μg/g)	847	4	0.90 (0.86–0.93)	0.82 (0.78–0.85)	103.48 (19.08–561.26)	20.47 (<0.001)	0.96 (0.02)	35, 37, 41, 43
Large studies—Crohn's disease *vs.* normal controls & IBS								
Adults (50 μg/g)	565	3	0.95 (0.91–0.97)	0.86 (0.81–0.89)	163.14 (29.96–888.41)	10.96 (0.012)	0.97 (0.02)	35, 37, 43

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; CRC = colorectal cancer; CI = confidence interval; DOR = diagnostic odds ratio; HG = heterogeneity; AUC = area under the curve; SE = standard error.

To resolve these issues, further prospective studies are required. An ideal study would focus on a single clinical question, *e.g.*, "determining the diagnostic precision of FC in distinguishing between IBD and IBS or functional bowel disorders in adults without red flag symptoms of IBD." It would report diagnostic precision results at different cutoff levels for FC positivity, and would be large enough to allow stratification of results by pretest probability of CD or UC (based on clinical assessment), as well as by disease severity and distribution.

If the findings of such future studies prove to be encouraging, FC could be an extremely useful tool for identifying patients who have IBD, and require urgent further investigation, from the pool of patients with IBS and nonspecific gastrointestinal complaints in a general gastroenterological out-patient practice. This particularly applies to the pediatric patient population, who require general anesthesia in order to undergo colonoscopy (9).

Based on the findings of a relatively low pooled sensitivity (36%) and specificity (71%) of FC in the diagnosis of colorectal neoplasia, FC cannot be recommended as a screening test for colorectal malignancies in the general population at present. Four of the included studies screened patients with a fecal occult blood test (FOBT) alongside FC (23, 32, 36, 41). Comparative sensitivity of FOBT for colorectal neoplasia ranged from 3% to 43%, with universally higher specificity at 90–97%. A large American study found that the combined use of a guaiac-based FOBT and an immunochemical FOBT had a sensitivity of 54% and specificity of 98% for colorectal neoplasia in an asymptomatic population (60). Whilst neither test is ideal for CRC screening, FOBT appears to be superior to FC for this purpose.

As FC correlates well with radio-labeled white-cell scanning as well as endoscopic and histological assessment of colonic inflammation in IBD, and is much cheaper and safer than both of these methods, it has potential as a noninvasive and objective way of monitoring disease activity in IBD. FC seems to have good diagnostic precision in predicting clinical relapse in CD and UC, and could therefore be used to target intensive medical therapy at patients at higher risk of relapse in the hope of reducing its occurrence. Some authors have recently argued that the optimal therapeutic end point in the treatment of CD should not be the remission of symptoms, but complete mucosal healing (9, 61), and FC could be used as a mucosal marker in this context.

In summary, this meta-analysis has shown that whilst FC cannot be recommended as a general screening test for CRC, it appears to have good diagnostic precision in distinguishing IBD from non-IBD diagnoses both in adults and children. The test seems to have better diagnostic precision for IBD at a cutoff of 100 μg/g than at 50 μg/g.

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STUDY HIGHLIGHTS

What Is Current Knowledge

- Fecal calprotectin (FC) has high sensitivity and specificity for the diagnosis of inflammatory bowel disease (IBD).
- FC may be useful in monitoring disease activity and prediction of relapse in IBD.
- FC has poorer diagnostic precision for colorectal cancer (CRC) than for IBD.
- Although the normal range for FC has been defined, an optimal cutoff point for distinguishing IBD from other diagnoses has not been identified.

What Is New Here

- This is the first study to quantify the overall diagnostic precision of FC for the diagnosis of IBD and cancer.
- FC appears to have a better diagnostic precision for IBD at a cutoff point of 100 μg/g than at 50 μg/g.

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CONFLICT OF INTEREST

Potential competing interests: None

Sources of funding: None

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How useful is fecal calprotectin for the diagnosis of IBD and colorectal cancer?

Original article von Roon AC *et al.* (2007) Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. *Am J Gastroenterol* **102:** 803–813

SYNOPSIS

KEYWORDS biomarker, colorectal cancer, diagnosis, fecal calprotectin, IBD

BACKGROUND

Fecal calprotectin is a biomarker for intestinal inflammation, but its accuracy for the diagnosis of IBD and colorectal cancer is uncertain.

OBJECTIVES

To perform a meta-analysis to determine the diagnostic accuracy of fecal calprotectin as a biomarker for IBD and colorectal cancer.

DESIGN AND INTERVENTION:

Ovid, EMBASE, MEDLINE and the Cochrane Database were searched to identify suitable studies to include in the meta-analysis. Search terms including "calprotectin and IBD", "calprotectin and Crohn's disease", "calprotectin and ulcerative colitis", "calprotectin and cancer", "calprotectin and intestinal inflammation", "IBD and leukocyte L1 complex" and "calprotectin and feces" were used to identify suitable studies. Eligible studies were prospective and compared the use of fecal calprotectin with histology for the diagnosis of Crohn's disease, ulcerative colitis and colorectal cancer. In addition, studies that compared fecal calprotectin with radiolabeled white cell scanning and clinical indices of disease activity for Crohn's disease and ulcerative colitis were included. Exclusion criteria included neonatal studies, studies that measured fecal calprotectin levels after drug, dietary or surgical intervention, and studies without a control group. Relevant data were extracted from eligible studies including the sensitivity and specificity of fecal calprotectin at various cut-off values. The quality of studies was assessed using the QUADAS (quality assessment for studies of diagnostic accuracy) tool. Analysis included the construction of summary receiver-operating characteristic curves and subgroup analysis.

OUTCOME MEASURES

The main outcome measures were the sensitivity and specificity of fecal calprotectin for diagnosis.

RESULTS

In total, 30 studies containing 5,983 individuals (including 663 with Crohn's disease, 361 with ulcerative colitis, 186 with IBD, 297 with colorectal cancer, 697 with IBS and 3,393 controls) were included in the meta-analysis. Fecal calprotectin levels were significantly higher (by 219.23 µg/g [95% CI 174.49–263.97]) in patients with IBD than in controls ($P<0.001$). The sensitivity and specificity of fecal calprotectin for the diagnosis of IBD was 0.95 (95% CI 0.93–0.97) and 0.91 (95% CI 0.86–0.91), respectively, with an area under the curve of 0.95. The accuracy of fecal calprotectin for the diagnosis of IBD was better in children than adults, and a fecal calprotectin cut-off level of 100 µg/g resulted in better diagnostic accuracy than a cut-off level of 50 µg/g. Fecal calprotectin levels were similar between patients with IBS and controls. Fecal calprotectin levels were higher in patients with colorectal cancer (by 132.2 µg/g [95% CI –59.18–323.56]) than in controls; however, this finding was not significant. The sensitivity and specificity of fecal calprotectin for the diagnosis of colorectal cancer was 0.36 (95% CI 0.34–0.39) and 0.71 (95% CI 0.70–0.73), respectively, with an area under the curve of 0.66.

CONCLUSIONS

Fecal calprotectin seems to be a useful biomarker for IBD at a cut-off level of 100 µg/g; however, it is not an accurate diagnostic test for colorectal cancer.

COMMENTARY

Bret A Lashner

No diagnostic test is perfect, even those considered as 'gold' standard. When deciding to incorporate a diagnostic test into clinical practice, the imperfections as well as the potential advantages of the test need to be carefully considered.[1] The costs of false-positive testing and false-negative testing, with their relative frequencies in a given population, need to be reviewed.

Let's examine such an accounting with regard to fecal calprotectin as a diagnostic test for colorectal cancer. Assume that fecal calprotectin will be used as a screening test to determine who should go on to colonoscopic evaluation. In such a situation, patients who test false-positive will have an unnecessary colonoscopy, and patients who test false-negative will not have their colorectal cancer diagnosed in a timely fashion. The penalties for false-positive testing seem much less severe than the penalties for false-negative testing in this situation; however, in a population at low risk for colorectal cancer, false-positive testing could lead to a vast number of unnecessary colonoscopies. Good screening tests need to have exceedingly high specificities and very good sensitivities.[2,3] The exact numbers can be debated, but as von Roon *et al.* show in their meta-analysis on the diagnostic precision of fecal calprotectin for IBD and colorectal cancer, a sensitivity of 36% and a specificity of 71% for fecal calprotectin as a colorectal cancer screening test are inadequate values. The authors of this meta-analysis properly conclude that fecal calprotectin should not be used as a screening test for colorectal cancer.

Fecal calprotectin might have a possible use for the diagnosis of IBD. This protein, however, will never be used as a sole test to diagnose this disease because radiological and endoscopic tests are vastly superior. The role for fecal calprotectin might be in its use as a complementary test to differentiate active inflammation from non-inflammatory conditions in a patient with IBD who experiences an acute exacerbation of symptoms. In such a patient, a false-positive test would lead to an unnecessary increase in anti-inflammatory therapy with concomitant exposure to toxicity and increased cost. A false-negative test would lead to a failure to introduce anti-inflammatory medication in a timely manner, with the resultant continuation of the patient's troublesome symptoms. In such a scenario, the sensitivity and specificity of diagnostic testing need not be exceedingly high, but should be high enough to minimize untreated disease and toxicity from medications. A sensitivity of 95% and a specificity of 91% for fecal calprotectin to identify inflammatory exacerbations of IBD would be adequate. von Roon *et al.* suggest that patients with IBD who have an exacerbation of symptoms might benefit from having fecal calprotectin testing performed before other diagnostic tests are carried out and before changes in therapy are initiated.

In conclusion, fecal calprotectin has a role in distinguishing inflammatory from non-inflammatory conditions that might account for an exacerbation of symptoms in patients with IBD; however, there seems to be little use for fecal calprotectin as a screening test for colorectal cancer.

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Competing interests
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PRACTICE POINT
Fecal calprotectin has a role in distinguishing inflammatory from non-inflammatory conditions in patients with IBD with symptoms of exacerbation; however, fecal calprotectin is not a useful screening test for colorectal cancer